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RAS PATHWAYS TO CELL CYCLE CONTROL AND CELL TRANSFORMATION

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1. ABSTRACT

Ras genes are among the most frequently activated oncogenes in cancer. The corresponding protooncogenes are proteins expressed in the majority of tissues in mammals and have a signal transduction activity. Ras proteins interact with a wide spectrum of regulators and downstream effectors producing different cellular responses, including proliferation, differentiation or apoptosis. This review deals with the most recent advances on the role of Ras in the signal transduction pathway from external signals to the cell cycle and gene expression control. We specially address the new developments on the effect of Ras activation in the regulation of different molecules driving the cell cycle progression. Both positive and negative regulators of the cyclin-dependent kinases (CDK), cyclins and CDK inhibitors, are targets of Ras, giving rise to different effects in the cell cycle progression. These Ras-mediated interactions are an

extraordinary example of the complexity of the signal transduction networks and the diversity of pathways used by Ras to propagate molecular signals.

2. INTRODUCTION

The Ras proteins have provided in the last 15 years a workhorse for normal and tumorigenic cellular signal transduction. These proteins are not only involved in a high percentage of human tumors but also are a central point for many signal transduction pathways in the cell. The analysis of these molecules has led to the understanding of the relationship between tumor development and signal transduction.

The *ras* genes were the first oncogenes to be implicated in human cancer. The experiments to isolate and characterize *ras* transforming genes were in many ways landmarks of molecular oncology. The *ras* oncogenes were initially discovered as the transforming genes of the Harvey and Kirsten murine sarcoma viruses (Ha-MSV, Ki-MSV) (1, 2). The molecular analysis of these retroviral genomes indicated that the rat sarcoma viruses contained genes not present in their parental murine leukemia viruses: genes that were transduced into the viral genome from the host cell. Analysis of the coding molecules showed proteins of 21 kDa (3). Subsequent work found that a non-transforming version of the *ras* genes is present in the eukaryotic genome and, remarkably, the normal and oncogenic forms differ by a single base pair change (4-6).

The presence of mutated alleles in human tumors emphasized the importance of the *ras* genes in the control of cellular proliferation. Basically, Ras proteins bind guanine nucleotides (GDP and GTP) with high affinity and posses intrinsic GTPase activity. The alternance between the inactive GDP-bound form and the active GTP-bound form is tightly regulated by different proteins and, thus, Ras proteins function as molecular switches in signal transduction. The analysis of their biological function has shown that Ras proteins are involved in transducing various extracellular signals including growth factor stimulation, T-cell activation, apoptosis, cytokine-induced proliferation and differentiation of hematopoietic cells, nerve growth factor-induced cell differentiation, development and regeneration of the nervous system, and inhibition of epithelial cell proliferation by transforming growth factor beta (TGFβ) (7-9). These signals enter in the cell through their appropriate receptors and are received by the membrane-associated Ras proteins, which send the signal to other locations in the cell through different downstream effectors.

Many extensive and intensive reviews have been published on *ras* genes and proteins (6, 7), and their role in human and experimental cancer (6, 10-13). In this review, we will only refer briefly to these aspects of Ras biology and we will focus on the new developments on the interactions of Ras with other effector molecules in the cell and, specially, on the effect of Ras on the regulators of the cell cycle. Several different pathways involved in Ras-dependent signal transduction have been shown to favor cell cycle progression or inhibition depending on the cellular context. These actions are determined by a complex network of proteins involved in many different areas of the cell biology.

3. Ras GENES and PROTEINS

3.1. Ras families

Ras proteins have served as a prototype for a group of 20-25 kDa guanine nucleotide-binding proteins that share structural homology, the superfamily of Ras-related proteins. This superfamily of proteins is comprised of over 60 mammalian members and, in terms of both primary sequence and biological activity, it can be conveniently divided into several subfamilies: Ras, Rho, Rab, Arf, Ran and Rad/Gem (14, 15). Many Ras-like proteins have also been identified broadly in eukaryotes including fungi, flies, frogs and nematodes. The Ras family consists of the Ras proteins (H-Ras, K-RasA, K-RasB and N-Ras), four Rap proteins (Rap1A, Rap1B, Rap2A and Rap2B), three R-Ras-like proteins (R-Ras, TC21 and R-Ras3), two Ral proteins (RalA and RalB), and the newly identified Rheb and M-Ras proteins (15-17). This family is characterized by high similarity in the effector domain, the so-called switch I region.

Several members of the Ras family have been found to be tumorigenic. The prototypic *ras* oncogenes H-, K- and N-ras are known to be oncogenically activated in a significant fraction of human tumors and its tumorigenic potential *in vitro* and *in vivo* has been extensively studied (6, 10-13). The R-*ras* genes, R-*ras*, *TC21* (R-*ras2*), and R-*ras3*, display transforming activity when mutationally activated (16, 18-21) and, in the case of *TC21*, mutations have been detected in some human tumor samples (19, 22). On the other hand, Rap1A (K-rev-1) was isolated due to its ability to reverse the transformation caused by the K-*ras* oncogene (23), an event thought to occur due to competition for common regulatory proteins. However, although it has been widely reported that Rap1 has an anti-Ras and anti-mitogenic activity, Rap1 has mitogenic and oncogenic properties in Swiss 3T3 cells, a system in which the Rap1 activator cAMP is known to be a positive regulator of cell growth (24).

Although all the members of the Ras family have GTPase activity, they are involved in many different cellular functions (14). Ras proteins are involved in the control of transcription, translation, cytoskeleton organization, Golgi trafficking and vesicle formation, and cell-cell junctions. M-Ras seems to be specially involved in the reorganization of actin cytoskeleton, similarly to the members of the Rho family, although it belongs to the Ras family based on the sequence homologies (17).

3.2. *Ras* genes and proteins in mammalian cells

3.2.1. Primary structure of *ras* genes

Mammalian cells encode three functional *ras* genes: H-*ras*, K-*ras* and N-*ras*, which show a very similar structure and function. They are composed of four exons and a 5' noncoding exon (exon ϕ) and differ widely in the intron sizes and sequences. The human genomic sequences span 3 kb (H-*ras*), 7 kb (N-*ras*) and more than 35 kb of genomic DNA (K-*ras*) and are located in different chromosomes. The K-*ras* gene differs slightly in having two alternative fourth coding exons (4A and 4B) giving rise to two isomorphic

proteins, K-RasA and K-RasB. These two forms of K-Ras diverge solely in their COOH-terminal 25 amino acids (6, 7).

The protein product of each vertebrate ras gene is approximately 21 kD in size, containing 188 (H-*ras*, K-*ras*A and N-*ras*) or 189 (K-*ras*B) amino acids. The remarkable degree of conservation between species as far apart in evolution as yeast and human strongly suggest that *ras* gene products play a fundamental role in key cellular processes. H-, K- and N-*ras* genes are found to be conserved in all mammalian species studied, suggesting that the duplications giving rise to the three *ras* genes occurred before the mammalian radiation. Thus, no differences in the amino acid sequence exist between the human and mouse H-Ras. Three differences are found in N-Ras (amino acids 168, 184 and 188), and 2 or 5 codons vary between the human and mouse K-RasA or K-RasB proteins (amino acids 131 and 187 in K-RasA, and positions 131, 181, 183, 185 and 186 in K-RasB). The evolutionary conservation of *ras* genes is underscored by their ability to function in heterologous systems. Thus, mammal *ras* genes can complement nonviable yeast mutants and induce phenotypic alterations in yeast cells. Similarly, some yeast RAS constructs are able to efficiently transform mouse NIH3T3 cells (6).

3.2.2. Gene Expression

The three *ras* genes carry promoters with high GC content that lack a TATA motif, this being a characteristic of housekeeping genes. Some regions controlling *ras* expression are found in the 5' region of the genes and in the first intron of the three *ras* genes (25, 26). Other elements at the 3' end of H-*ras* have been described that may also contribute to its expression (7). For instance, a negative-acting element in the last intron of human H-*ras* produces alternative splicing suppressing $p21^{H\text{-}Ras}$ expression (27). The alternative H-*ras* protein, named p19, displays properties of a negative regulator of Ras (28). Mammalian *ras* genes are expressed in all cell lineages and organs, although there are some differences in the level of expression of each of these genes in embryonic development and certain adult tissues (29, 30). K-rasA shows a more restricted pattern of expression (31). The different pattern of expression of the four Ras proteins suggests a functional difference, however, at least one *ras* gene is expressed in all cell types.

The levels of *ras* mRNA seem to be basically constant in the cell, being the Ras activity regulated by the binding of GDP or GTP, a process catalyzed by different Ras regulators (see below). Ras protein activity is known to be induced by serum and about 0.3 - 5% (depending on the assay used) of the 20,000-30,000 Ras protein molecules per cell are in the active GTP-bound form in NIH 3T3 cells supplemented with calf serum (7, 32). However, not only activity but also expression of the three *ras* genes has been shown to slightly increase in a serum-dependent manner (33). In addition, a cross-regulation in the transcriptional induction of the *ras* genes has been also described, this induction being dependent on an element located in a non-promoter area (34). In the case of N-ras, an additional regulation may arise from its genomic structure being located immediately downstream of another gene: *unr* (*u*pstream *N*-*r*as). N-*ras* constitutes with *unr* a tightly linked tandem of ubiquitously expressed genes. Recently, the upstream gene *unr* has been reported to down-modulate N-*ras* expression *in vivo* (35) since deletion of the *unr* promoter by homologous recombination in murine embryonic stem cells revealed an increase in N-*ras* mRNA, indicating that transcription of *unr* can negatively regulate that of N-*ras* by transcriptional interference.

3.2.3. Posttranslational modifications

Ras proteins are synthesized on free ribosomes in the cytoplasm and have a half-life of at least 24 h (36). These proteins become associated with the inner side of the plasma membrane after posttranslational modifications. These modifications are required for Ras activity, since activated *ras* genes lose their transforming activity with mutations that render the protein cytosolic (37). Transforming activity can be rescued by placing a membrane-anchoring domain at the N-terminus of Cys186 mutants (38, 39). The sequences at the C-terminus are essential for membrane association and the conserved Cys186 in the CAAX motif is required to initiate the posttranslational modifications. This cysteine is first modified by a C_{15} polyisoprenyl (farnesyl) moiety attached to it. Subsequently the three C-terminal amino acids to Cys186 are proteolytically removed and the newly generated farnesylated Ras C-terminal cysteine is carboxymethylated, leading to a more hydrophobic protein with higher affinity for membranes. H-Ras, N-Ras and K-RasA have in addition a palmitoyl group added to a cysteine in the hypervariable region near the C-terminus (7, 40).

3.2.4. Protein structure and biochemical function

Ras proteins contain five noncontiguous domains required for Ras function: residues 5-63, 77-92, 109-123, 139-165, and the carboxyl terminal sequences Cys186-A-X-COOH (6). Figure 1 shows the location of these domains and their functional significance. At least five noncontiguous domains have been shown to be dispensable for the *ras* oncogenes transforming properties, including the amino terminal end, three internal domains (residues 64-76, 93-108, and 124-138), and the hypervariable region. However, whether these regions are completely dispensable for the normal function of *ras* protooncogenes is not clear (6).

Ras proteins function as guanosine diphosphate/guanosine triphosphate (GDP/GTP) regulated switches positioned at the inner surface of the plasma membrane to transduce extracellular ligand-mediated stimuli into the cytoplasm. Ras biological activity is controlled by a catalyzed GDP/GTP cycle. Most Ras molecules in the cell exist in their inactive state, characterized by a conformation that allows binding to GDP. The exchange of GDP by GTP is followed by a conformational change of the Ras protein to its activated state. Active Ras proteins are then able to interact with effector molecules and propagate the signal. The hydrolysis of GTP to GDP returns the active Ras protein to the inactive GDP-bound state. This hydrolysis can be accomplished by the Ras intrinsic GTPase activity. However, this intrinsic GTPase activity is very low and a faster hydrolysis of GTP is catalyzed by other molecules.

There are hence two types of Ras regulators, those promoting the formation of the active GTP-bound state-referred to as Guanine nucleotide Exchange Factors (GEF) or

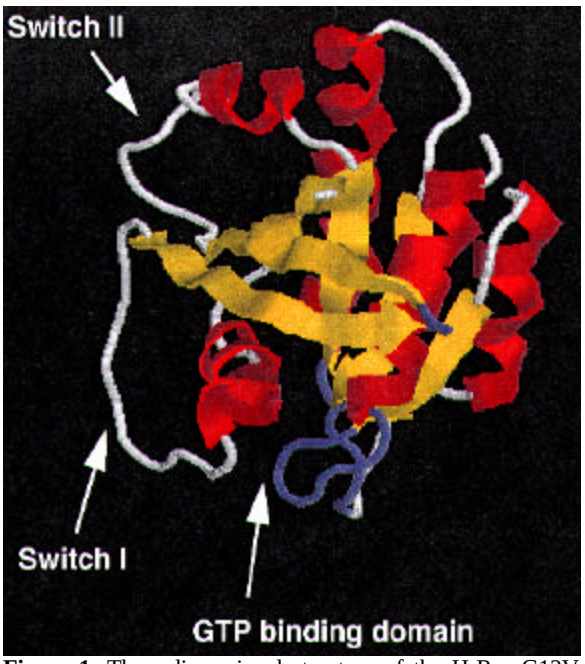

Figure 1. Three-dimensional structure of the H-Ras G12V mutant showing the switch I and II domains, two hydrophilic regions exposed to the effectors in the external surface of the protein. These regions are close to the guanine nucleotide-binding domain and change their conformation after GTP/GDP exchange. This figure was ellaborated using RasMac 2.6 and the entry #521P (41) from the Protein Data Bank (Brookhaven National Laboratory).

Guanine Dissociation Stimulators (GDS)—, and those promoting the formation of the inactive GDP-bound state-referred to as GTPase Activating Proteins (GAPs). GEFs act as positive regulators or activators of Ras, whereas GAPs inhibit the Ras-mediated signal transduction (42, 43).

The three-dimensional structure of GDP- or GTP-bound Ras proteins provides an explanation of the GTP cycle and its regulation by GEFs or GAPs (41, 42, 44). Ras proteins bind guanine nucleotides with high affinity and at least four motifs are involved in this binding: residues 10-18, involved in the binding to the α- and β-phosphates, residues 57-63, involved in binding to the Mg^{2+} and to the γ-phosphate in the GTP-bound form, and regions involving residues 116-119 and 144-147, both of them important for the binding of the guanine ring (7). However, the main differences between the GTP-bound and GDP-bound forms appear to be limited to two loops of the protein: the switch I, encompassing residues 30-38 (loop 2), and the switch II, residues 60-76 which consist of helix 2 and loop 4 (45, 46). These regions contain hydrophilic residues located in the external surface of the molecule. The switch regions are close to the γ-phosphate group of GTP and exhibit different conformations depending upon whether GDP or GTP is bound. The switch I region is the main effector binding site (aa 32-40) responsible in part for interactions with GAPs and downstream effectors. The interactions of GEFs with Ras is, in part, with the switch II region. Thus, the conformational changes of these switch regions in the GTP cycle are involved in the binding to regulatory proteins and in the transduction of the signal to downstream effectors.

3.3. Ras activation and transforming properties

Ras genes are activated in up to 30% of human tumors (10, 11) and are frequently mutated in experimental models in animals (12, 13). K-*ras* mutations have been found preferentially in adenocarcinomas and are common in pancreatic cancers, cholangiocarcinomas, colorectal malignancies and in adenocarcinoma of the lung. H-*ras* mutations can be found in cutaneous squamous cell carcinomas and in squamous head and neck tumors. Finally, N-*ras* mutations are found most frequently in acute leukemias (mainly of the myeloblastic cell type) and in the myelodysplastic syndromes (11). Activated *ras* genes have been detected in premalignant lesions as well, suggesting a potential role in tumor initiation. It is believed that *ras* mutations may arise during the lifespan of healthy individuals and may remain silent until other genetic alterations occur, as it has been documented in the multistep model of colon carcinogenesis (47).

Oncogenic mutants of Ras constitutively remain in the active, GTP-bound form. NIH 3T3 cells transformed by activated Ras oncogenes contain 29% of the Ras protein in the GTP-bound form compared to < 0.3% in the same cells containing the protooncogene (32). Activating mutations all lead to two major biochemical changes in the Ras protein: impaired GTPase activity or facilitated GDP/GTP exchange, depending on the location of the mutation. Impaired GTPase activity is associated with mutations at positions 12, 13, 59, 61 and 63. These mutations reduce the intrinsic GTPase activity of the protein and render the protein resistant to GAP activity by p120GAP and NF1. Increased guanine nucleotide exchange results from mutations at positions 16, 17, 116, 117, 119, 144 and 146 (7, 42).

In addition to point mutations, *ras* genes can also be activated by overexpression in the absence of mutations. Thus, in NIH 3T3 cells overexpressing the H-*ras* protoncogene, the percentage of GTP-bound active Ras is still < 0.3% as in the parental cells; however, due to the overexpression, the amount of Ras-GTP proteins per cell increases from 1.3 fmol to 21.3 fmol per mg of cellular protein (32). The transcriptional activation of normal *ras* genes has been shown to also contribute to the development of some tumors and to play a role in the transforming properties of some cell lines. In these cases, the insertion of retrovirus close to the *ras* genes produced high levels of non mutated Ras proteins (6). In addition, overexpression of N-*ras* in transgenic mice is associated with tumorigenesis without mutations in any of the critical codons (48). Quantitative analysis have estimated that the levels of *ras* transcripts in about 50% of human tumors are 2- to 10-fold higher than in control tissue (6). Recent studies have also shown that a 100-fold increase in *ras* expression is necessary to transform cells *in vitro*, and oncogenic *ras* is not able to produce phenotypic transformation when expressed under this threshold (49).

3.4. Differences between H-, K- and N-Ras

Although the three *ras* genes and proteins are similar in structure and in function, there are several differences between the mammalian Ras isoforms:

3.4.1. Genetic and Structural Differences

1. The first 85 amino acids of N-, H-, and K-Ras are identical and the next 80 amino acids exhibit an 85% homology between any pair of Ras isoforms. However, Ras proteins differ substantially in the amino acidic composition of the C-terminus (50). The regions which encode unique sequences are highly conserved between different mammalian species (6). These sequence differences, for instance, render K-*ras* but not H-*ras* or N-*ras* susceptible to protein kinase C (PKC) phosphorylation (51).

2. The expression pattern of each *ras* gene is slightly different according to the organ (29, 52-54) and during development (29, 55) and differentiation (56). In addition, K-*ras*A has a distinctive pattern of expression in embryonic and adult tissues (31).

3. Protein maturation of Ras products displays some differences among the genes: K-RasB has a polybasic domain in its C-terminal and is geranylgeranylated, but H-Ras and N-Ras are not (57). Furthermore, all the three Ras proteins exhibit differences in their post-translational modification (58). These different modifications produce differences in the response of the three Ras proteins to farnesyltransferase and geranylgeranyltransferase I inhibitors (59, 60).

3.4.2. Functional Differences

1. Different *ras* genes are activated in different human tumors suggesting cell-specific activities (10, 11). For instance, K-*ras* is preferentially activated in colon and pancreas carcinomas, H-*ras* in bladder and kidney carcinomas, and N-*ras* in myeloid and lymphoid disorders.

2. H-*ras* has the unique ability to inhibit c-*fos* induction by the protein kinase C activator TPA (61).

3. Although p120-GAP binds to both N-Ras and H-Ras with comparable affinity, NF1-GAP has a fourfold higher affinity for H-Ras than for N-Ras (62).

4. Among the members of Ras-guanine exchange factors, SmgGDS is active on K-Ras but not on H-Ras (63). On the other hand, Ras-GRF (Cdc25Mm) activates H-Ras but not N-Ras or K-RasB proteins *in vivo* (64).

5. Differences in the mechanism by which K-Ras and H-Ras inhibit expression of proteins associated with thyroid differentiation have been reported (65). Whereas K-Ras reduces expression of thyroid transcription factor-1 (TTF-1), H-Ras acted distally to antagonize the action of TTF-1 on its target genes.

6. The three *ras* genes show different transforming potential depending on the cell type. Oncogenic H-*ras* is at least 10 times more transforming in rat-2 and NIH 3T3 fibroblasts, whereas oncogenic N-*ras* is more transforming in human hematopoietic TF-1 cells and in the murine hematopoietic cell line FDC-P1 (66). Thus, the N-*ras*12 mutant, but not the K- or H-*ras* mutants, leads to growth factor autonomy of the IL-3/granulocyte macrophage colony-stimulating factor-dependent human myeloid cell line TF-1. These effects reside in unique sequences between amino acids 84-143 of H-Ras or in the final third of the N-Ras protein (66).

7. N-*ras*-specific antisense oligomers can inhibit granulocyte/macrophage-colony formation *in vitro* of normal hematopoietic cells exposed to growth factors (IL-3, GM-CSF, M-CSF) (67), suggesting that N-Ras is the main responsible for this ability. On the other hand, it has been demonstrated that K-*ras*, but not H-*ras*, contributes to the proliferation of normal human lung fibroblasts (MCR-5 cells) (68).

8. In the IL-6-dependent myeloma cell line ANBL6, N-*ras*12 and N-*ras*61 suppress apoptosis in the absence of IL-6, whereas K-*ras*12 does not (69).

9. Oncogenic K-*ras*, but not oncogenic H-*ras*, blocks glycosylation of β1 integrin and up-regulates the embryonic adhesion protein carcinoembryonic antigen (CEA), disrupting cell adhesion to collagen I and laminin, and preventing basolateral polarity and subsequent differentiation of colon epithelial cells (70, 71).

10. Mice knockout for the different *ras* genes show different responses. N-*ras* knockout mice develop and reproduce normally (72). H-*ras* knockout animals are also normal (E. Santos, personal communication), whereas K-*ras* seems to be essential for the development of the mouse embryo (73, 74). H-Ras and N-Ras show different *in vivo* affinity for Raf1. Thus, Raf1 has been reported not to be involved in the Erk activation by H-Ras although is the direct target of N-Ras for the activation of this pathway (75).

A more complete understanding of the Ras function is being provided by the finding of cellular molecules interacting with Ras. A wide range of molecules with different cellular functions have been shown to interact with Ras, upstream or downstream, in the signal transduction pathways. These molecules are reviewed in the following sections.

4. UPSTREAM OF Ras

4.1. Signal transduction from external factors to Ras

Ras is a point of convergence of many signaling pathways from a diverse array of extracellular signals, such as growth factors, cytokines, hormones and neurotransmitters that stimulate cell surface receptors. Basically, extracellular signals that activate Ras proteins can be mediated through different types of receptors: tyrosine kinase receptors (*e.g.* platelet derived growth factor (PDGF) receptor or epidermal growth factor (EGF) receptor), cytokine receptors (*e.g.* IL-2 receptor), T cell receptors, and subunits of heterotrimeric G proteins. In mammalian cells, Erk activation from tyrosine kinase receptors is the most studied route of the Ras transduced pathways. Many Src Homology 2 (SH2)-containing proteins bind to the phosphotyrosine residues of the activated tyrosine kinase receptors, including Grb2, Shc, PI3K, phospholipase C-γ (PLC-γ), p120-GAP and SH-PTP2 tyrosine phosphatase (Syp) (76, 77). Both Grb2 and Shc proteins have been demonstrated to contribute to Ras activation through the direct binding between Grb2 and Sos and the subsequent activation of the Ras exchange factor Sos (see below).

Heterotrimeric guanine triphosphate-binding proteins (G proteins), can stimulate the Ras/Erk pathway through the Gβγ subunits. This stimulation is inhibited by wortmannin (a PI3K inhibitor), whereas stimulation of this

pathway by tyrosine kinase receptors such as the epidermal growth factor receptor is insensitive to wortmannin (78). This suggests a role for PI3K in Ras activation by heterotrimeric G protein-coupled receptors. This effect is dependent on PI3Kγ, a PI3K isotype activated by both the α and βγ subunits of heterotrimeric G proteins (78, 79).

A different intriguing example is the MAP kinase pathway activation by protein kinase C. By using the Ras dominant negative, Ras17N (80), this activation has been shown to by Ras-dependent in some cell types such as NIH 3T3 cells (81) but Ras-independent in a number of other cell types (82) Recently, Raf-1 activation by PKC has been shown to be mediated through Ras activation. However, this activation differs from activation by receptor tyrosine kinases in that it is not blocked by Ras17N, the "dominant negative" form of Ras, in COS cells (82). These results indicate that Ras is a component of PKC signaling and that the absence of an effect of Ras17N expression cannot be used to conclude that Ras is not part of a signaling pathway in a specific cell type. However, since Raf-1 seems to associate *in vivo* preferentially with N-Ras but not H-Ras (75), the role of the three isoforms in PKC signaling can be different. In fact, cells deficient in N-Ras, but not in the other Ras isoforms, seem to have a decreased response to the PKC-dependent PMA stimulation (M. M. & A. P., unpublished data), suggesting an important role for N-Ras in PKC signaling.

4.2. Ras activation: Nucleotide exchange factors for Ras

In order to facilitate the exchange of GTP for GDP, the Guanine Exchange Factor (GEF) must first form a complex with the GDP bound form of the GTPase. GDP is then released from the complex and the high GTP/GDP intracellular ratio ensures that the released nucleotide is immediately replaced with GTP. GEF then dissociates leaving the Ras in the active form (14, 83). Various GEFs have been isolated for the Ras subfamily members, such as Sos, RasGRF, RasGRF2, RasGRP, C3G, and RalGDS-related sequences (43, 84-86). A mammalian GEF, related in sequence to the *Saccharomyces cerevisiae* Ras exchange factor CDC25, was isolated first and named RasGRF (or Cdc25Mn). The related proteins Sos1 and Sos2 are most closely related to the *Drosophila son-of-sevenless* product. Sos is known to stimulate the GTP exchange on Ras (87), while GRF has been shown to activate both Ras (88) and R-Ras (89). C3G has recently been shown to possess exchange activity on both K-rev-1/Rap1A and R-ras (89). Finally, RalGDS-related sequences include a growing family of proteins with exchange activities for the Ral GTPases. Interestingly, most of these RalGDS proteins have been shown to be putative Ras effectors (see below).

Only some of these proteins (Sos, RasGRF, RasGRF2, RasGRP, Vav, C3G and SmgGDS) have been reported to act as exchange factors for the true Ras proteins, although Vav and C3G rather seem to act *in vivo* as exchange factors for other GTPases than Ras. Their properties are discussed below.

4.2.1. Sos

The Sos1 and Sos2 proteins are widely expressed in mouse and human tissues and are related to the *Drosophila* son-of-sevenless product, which functions upstream of Ras in the R7 cell development of the Drosophila eye (90, 91). A central region of about 200 amino acids in the mSos1 protein is similar to a comparable region of Cdc25, Sdc25 and Ste6 (43). This domain is known to catalyze exchange activity on H-Ras and N-Ras, but not RalA or Cdc42 (92). The C-terminal proline-rich region of Sos binds to the Src Homology 3 (SH3) domains of Grb2 and the Sos-Grb2 complex interacts with the activated tyrosine kinase receptors, as the epidermal growth factor (EGF) receptor, through the SH2 domain of Grb2, thus linking external growth factor-tyrosine kinases signaling and Ras activation (93-97).

In addition to the Cdc25 domain, responsible for the Ras-GEF activity, and the Grb2-binding domain, Sos proteins possess Dbl homology (DH) and pleckstrin homology (PH) domains arranged in tandem in the N-terminal region. DH domains have been demonstrated to promote guanine nucleotide exchange on Rho family GTPases, whereas PH domains participate in protein-protein and protein-lipid interactions, binding tightly and specifically to phosphatidylinositol derivatives. Both the Grb2- and the DH-binding domains have been recently shown to have autoinhibitory effects on the intrinsic GEF activity of the Cdc25 domain (98). The DH domain of Sos is able to stimulate guanine nucleotide exchange on Rac but not Cdc42. Both the exchange activity on Rac and Ras are inhibited by binding of the PH domain to phosphatidylinositol-4,5-biphosphate (PtdIns[4,5]P2), a substrate of the phosphatidylinositide 3-kinase (PI3K) and this activity is regained when coexpressed with activated Ras (99, 100). Thus, Sos may couple Ras and Rac activation through its different domains and this interactions may be dependent on PI3K signaling.

In addition to tyrosine kinase-dependent activation of the Sos-Ras pathway, a different mechanism seems to be involved in the PKC-dependent activation of Ras. In NIH 3T3 cells, the PKC-dependent activation of the Ras-Erk signaling pathway by PMA is suppressed by the dominant negative Raf1 and, in some cell types, also by the dominant negative forms of Ras and Sos. The PMA signal seems to be fed at or upstream of Shc and involves serine (but not tyrosine, as it occurs in the growth factor-induced Shc phosphorylation) residues (81).

4.2.2. RasGRF

This protein was identified as homologous to the *Saccharomyces cerevisiae* Ras exchange factor encoded by CDC25 (101, 102). RasGRF, also named Cdc25Mm, contains Dbl homology (DH) and pleckstrin homology (PH) domains and is expressed specifically in brain. Both GRF and Sos are expressed in several different protein forms by alternative splicing. The heterogeneity of GRF and Sos products may therefore contribute to a fine regulation of Ras activation in different tissues or at different stages of development (103). RasGRF has been recently shown to activate H-Ras but not N-Ras or K-RasB proteins *in vivo* (64).

4.2.3. RasGRF2

RasGRF2 is a new multidomain protein catalyzing the specific release of GDP from Ras but not other GTPases

(85). This protein contains a Cdc25-related domain in the COOH terminus of the protein. In the NH_2-terminal region, it contains several domains with homology to various signaling proteins including two pleckstrin homology domains, a Dbl homology region, and an IQ motif required for its apparent constitutive association with calmodulin. Accordingly, calcium influx caused a shift of RasGRF2 subcellular localization from cytosolic to the cell periphery, stimulating GTP binding by Ras and potentiating calcium ionophore-induced activation of Erk (85).

4.2.4. RasGRP

This recently isolated member of the Ras nucleotide exchange factor family is characterized by an atypical pair of "EF hands" that bind calcium and a diacylglycerol (DAG)-binding domain. RasGRP activates Ras and the Erk pathway in response to DAG through the DAG-binding domain. This exchange factor is expressed in the nervous system suggesting a role in the coupling of changes in DAG and calcium concentrations to Ras activation (86).

4.2.5. Some considerations on SmgGDS, Vav and C3G

Some other exchange factors have been thought to participate on Ras activation. Although Vav has similar domains to the Dbl family proteins, unexpectedly, a GEF activity specific for Ras rather than Rho subfamily members was described (reviewed in Ref. 104). Several observations have shown, however, that Vav is not a true exchange factor for Ras. The morphological changes that are observed in Vav-expressing NIH 3T3 cells are distinct from those seen when the same cells are expressing other Ras GEFs such as RasGRF, suggesting that the Vav-associated oncogenic activity may not be a consequence of Ras GEF activity (105). Moreover, no increase in the level of Ras-GTP is detected in cells transformed with Vav (106). Recently, Vav protein has been shown to bind and stimulate GDP release from Rac1 and Cdc42, and to a lesser degree RhoA, but not from Ras (107, 108). This lack of Vav protein exchange on Ras is in agreement with the earlier results indicating a Ras-independent transformation by oncogenic Vav (105). Importantly, Vav has been shown to play an important role on Rac activation regulated by the substrates and products of phosphoinositide 3-kinase (PI3K) (109).

C3G is an exchange factor which contains a Cdc25 homology domain and complements yeast cells with the cdc25 mutation; however, it has been shown to be a specific GEF for the Ras-related protein Rap1. On the other hand, SmgGDS is a protein that catalyzes exchange on K-Ras4B and other Ras-related GTPases, but not H-Ras (63). The relative contribution of these proteins to Ras regulation remains to be clarified.

4.3. Ras inactivation by GAPs

The Ras GTPase activity results in the slow hydrolysis of bound GTP, leaving the protein complexed with GDP. However, the intrinsic rate of dissociation of the Ras-GTP complex is very low, approximately 10^{-5} moles per sec dissociating per mole of complex, and since the intracellular GTP concentration is much greater than that of GDP, it seemed clear that additional regulatory proteins should be involved in the Ras GTPase activity. A cytosolic protein fraction was found to be able to stimulate the hydrolysis of GTP bound to Ras (110, 111). A 120 kDa protein, named p120-GAP for *G*TPase *A*ctivating *P*rotein, was initially purified and the corresponding cDNA cloned. This protein binds preferentially to Ras-GTP and this binding is impaired in some Ras effector mutants.

At least six different GAPs have been found to date in mammals: p120-GAP, the neurofibromatosis type 1 protein NF1 (112, 113), GAP1m, GAP1IP4BP (114), IQGAP1 (115, 116) and SynGAP, a Ras GAP selectively expressed at excitatory synapses in the brain (117, 118). All these proteins negatively regulate Ras through their GTPase stimulatory activities. Accordingly, overexpression of GAP is associated with a reduction of endogenous Ras in the GTP-bound form and can prevent transformation by overexpression of normal Ras or by other oncogenes whose transforming activities depend on endogenous Ras (42, 119). However, GAP does not affect to the transforming potential of some Ras mutants, such as those with mutations in codons 12, 13 or 61. Analysis of these mutants showed that mutations in the effector domain of Ras render the protein unable to respond to GAP, and they are therefore constitutively in the GTP-bound form (7, 44). In addition to their GTPase stimulating activity, a putative effector role for p120GAP and NF1 has been suggested (see below).

5. DOWNSTREAM OF Ras

The activated Ras-GTP complex transmits the signal downstream the pathway by interacting with target proteins. In the last few years, a wide spectrum of proteins have been shown to interact with the GTP-bound form of Ras and the list of potential Ras effectors is still growing (120, 121). The binding of effector proteins involves interactions with the effector loop (amino acids 32-40), some flanking residues, and the switch II region (amino acids 60-72) (122-127). These interactions are possible after the exchange of Ras-GDP by GTP, thereby rendering a conformational change wherein two discrete Ras segments, switch I (aa 32-40) and switch II (aa 60-72) exhibit a significant displacement as compared with the GDP-bound state. In addition, the residues 26, 31, 41, 45, 46, 48, 49, and 53, flanking the effector region, have been found to be critical for the Ras signaling even though they do not change their conformations upon ligand exchange from GDP to GTP. These residues, therefore, were designated the "activator region" (128) or "constitutive effector region" (124). The specific binding of the different effectors to Ras and the amino acids involved is now being studied using a wide panel of different effector mutants of Ras (129-131).

To date, several different mammalian proteins have been demonstrated to bind Ras in a GTP-dependent manner through the effector domain loop: Raf proteins, members of the RalGDS family, PI3K, MEKK1, Rin1, AF-6, PKC-ζ, Nore1, etc. (figure 2). All these proteins are classified as Ras effectors based upon two essential paradigms: (1) the effector proteins bind preferentially to the activated GTP-bound form of Ras and (2) all of these proteins interact with some region in the so-called effector domains of Ras and this binding is impaired in some of the effector loop mutants. The interaction

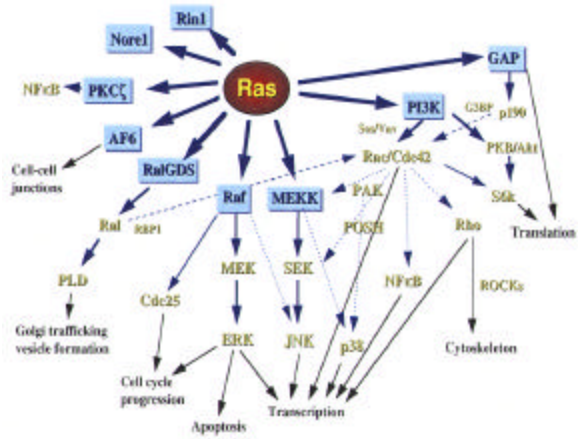

Figure 2. Ras effectors and downstream pathways.

between Ras and the effector molecule should elicit activation of the effector or production of a biochemical effect on downstream targets. Finally, a functional knockout of the putative effector should abolish part of the Ras-mediated signal (128). The following paragraphs describe some experimental data on the possible function of these effectors in the Ras-dependent signal transduction.

5.1. Raf and the MEK/Erk pathway

The first Ras effectors to be identified in mammalian cells were the protein kinases of the Raf family. The ability of Raf to bind Ras in a GTP-dependent manner *in vitro* and *in vivo* is the cardinal biochemical evidence in support of a direct effector role for Raf (132-135). GTP-bound Ras binds cytoplasmic Raf-1 and translocates it to the plasma membrane where Raf1 kinase becomes activated by a mechanism which is still poorly understood but is Ras-independent (136). Activated Raf phosphorylates MAP kinase-extracellular signal-regulated kinase (MEK) (137, 138) which in turn activates the p42 and p44 MAPK/Erk kinases (139-142). Unlike Raf and MEK, the Erks have numerous substrates including p90 ribosomal S6 kinase, cPLA2 and PHAS-1 (120). Erk phosphorylation promotes its homodimerization (143) and results in the translocation of the Erks into the nucleus leading to the activation by direct phosphorylation of transcription factors, such as $p62^{TCF}$/Elk-1 and Ets-2. These factors are involved in ternary complex formation at the serum response elements (SRE) (144), which regulate the expression of immediate-early genes, such as the c-*fos* and HB-EGF genes and eventually to cell proliferation (145, 146). Erks, in addition, mediate gene expression through the phosphorylation and activation of $p90^{RSK2}$, which phosphorylates CREB to activate its transactivation potential (147). The central role of the Erks in signal transduction is underscored by the ability of constitutively activated MEK protein kinase to mimic the effects of Ras activation in some cell types (138, 148).

Raf, through the Erk cascade, is a key effector pathway of Ras, as supported by considerable evidence. For instance, constitutively activated mutants of Raf1 cause a transformed and tumorigenic phenotype similar to that caused by Ras in rodent fibroblasts (149). Transformation of fibroblasts by upstream activators of Erk is blocked by dominant negative forms of MEK1, establishing that activation of Erk is required for transformation. However, as described below, Ras has other effectors which play important roles in Ras transformation. Indeed, it should be noted that transformation by activated MEK1 is blocked by microinjected Ras antibody showing that direct activation of Erk targets is not sufficient for transformation (138). Thus, some of the Ras induced changes could be caused by factors secreted by the cells which act through receptors coupled to Ras along pathways distinct from the Raf/Erk pathway. In fact, some mitogenic factors are released in Ras-transformed cells that act as autocrine modulators of cell growth (150).

Mammalian cells contain three Raf genes encoding Raf-1 (c-Raf), A-Raf and B-Raf, and B-Raf exists in multiple spliced forms. A notable difference between B-Raf and Raf-1 is the absence of two tyrosine phosphorylation sites that are involved in the Ras-dependent activation of Raf-1 by tyrosine kinases, suggesting a different regulation for these proteins (151). Thus, Raf-1 and A-Raf have been reported to be weakly activated by oncogenic Ras and more strongly activated by oncogenic Src. B-Raf by contrast seems to be strongly activated by Ras and not by Src (151). Ras activation of B-Raf seems to occur by induction of a conformational change that results in B-Raf activation, rather than recruitment to the membrane, as in the case of Raf1 (121, 152, 153). All these studies, in general, used only the oncogenic form of H-Ras and did not compare with the other Ras members. Other authors have reported recently that Raf-1 is weakly activated by H-Ras *in vivo* but strongly binds and is activated by N-Ras (75). Both Ras isoforms activate the MAP kinase pathway suggesting different Ras-dependent mechanisms for activation of this pathway.

A number of other proteins with different cellular functions are connected with the Ras-dependent Raf-MEK-Erk pathway. One of the proteins involved in the Erk pathway regulation is Tpl2/Cot. A C-terminally truncated form of this protein was isolated as an oncogene in a transfection assay and, independently, in Moloney murine leukemia virus-induced T cell lymphomas in rats. Tpl2/Cot is also a protein-serine kinase related to the Raf family and has been shown to phosphorylate and activate the MEK1-Erk and JNKK-JNK/SAPK pathways in a Ras- and Raf-dependent manner (154-156). The 14-3-3 proteins have been shown to associate with the products of several proto-oncogenes, such as Raf-1 among others. At least seven mammalian 14-3-3 isoforms have been identified. These proteins have been shown to interact with Raf-1 in the yeast two-hybrid system and in binding assays, and this interaction does not interfere with the binding between Raf-1 and Ras. The functional significance is not clear, but 14-3-3 together with the Hsp proteins appear to be involved in the modulation of Raf-1 activity (40, 157). Recently, 14-3-3 has been found to be bound to Raf-1 in the cytosol being totally displaced when Raf-1 is recruited to the plasma membrane by Ras. *In vitro* and *in vivo* assays show that 14-3-3 may be important to facilitate the Ras-dependent Raf-1 activation in the plasma membrane and recycling to the cytosol (158).

Ksr (kinase suppressor of Ras) is an additional player in the Ras-MAP kinase pathway, although its precise

role in Ras signaling is not yet well defined. Ksr was isolated as a regulator of the Ras-MAP kinase pathway by genetic screens in *Drosophila* and *Caenorhabditis elegans*. In these organisms, mutations in Ksr resulted in attenuation of Ras-mediated signaling. The mammalian Ksr inhibits Erk activation by insulin, phorbol ester, activated alleles of Ras, Raf and Erk, and is also able to block Ras-induced transformation (159, 160). Ksr shows homology to the Raf family of kinases and, recently, a direct interaction of Ksr with both MEK-1 and MEK-2 activators of Erk and also with the Erk proteins has been reported (160, 161). Thus, Ksr has been proposed to control MAP kinase signaling as a scaffold protein that links MEK and its substrate Erk (161). On the other hand, a kinase-deficient mutant of Ksr functions as a dominant interfering mutant which elevates phosphorylation of Elk-1, a member of the TCF family, and Elk-1 dependent transcription. Since Ksr blocks the activation of TCF without affecting the activation of MAP kinase itself, it seems capable to uncoupling the MAP kinase activation from its target phosphorylation, providing a novel mechanism for modulating the MAP kinase signaling pathway (162).

The serine and threonine protein kinase cascade consisting of Raf, MEK, and Erk is thus one of the best-characterized Ras effector systems. This pathway has been shown to be physiologically involved in a wide spectrum of Ras-dependent cell processes and is an important one in Ras transformation. However, other Ras downstream pathways participate in Ras signaling as described below.

5.2. RalGDS family and the Ral cascade

RalGDS (Ral GDP Dissociation Stimulator), a nucleotide exchange factor for Ral, was shown to be a Ras effector by several groups (163-166). Several other RalGDS-related proteins have also been found to bind to the GTP form of Ras: Rgl (RalGDS-Like; 164), Rgl2 (167), and Rlf (RalGDS-like factor; 168). All these proteins are nucleotide exchange factors for a GTPase member of the Ras family, Ral, and, in addition, they contain an 80 to 100-residue fragment at the carboxy-terminal end that binds to Ras. RalGDS interacts with Ras in mammalian cells in response to extracellular signals (169, 170) and RalGDS has been shown to stimulate the GDP/GTP exchange of Ral in a Ras-dependent manner in intact cells (171). Stimulation of various growth factor receptors results in rapid activation of Ral, correlating with and being dependent on Ras activation (172). Colocalization of Ras and Ral on the membrane has been shown to be required for Ras-dependent Ral activation through RalGDS (173). Dominant negative mutants of Ral can suppress oncogenic transformation by Ras in 50-75% (171, 174) indicating that Ral activation by Ras-RalGDS mediates some of the downstream effects of Ras on cells. These RalGDS-family proteins therefore are responsible for the transmission of part of the signals from Ras to Ral or other proteins. Structural studies of the interaction of Ras with RalGDS have shown that both the switch I and II regions are involved showing different orientations and side chain interactions than the complexes with Raf (175). These different interactions may account for the specificity with which Ras distinguishes the two effectors. In addition, cyclic AMP-dependent protein kinase (protein kinase A) can be physiologically involved in regulating the selectivity of Ras binding to either RalGDS or Raf1 (169).

Potential downstream targets for Ral proteins include a Cdc42/Rac GTPase-activating protein and a phospholipase D. A putative effector of Ral, Ral interacting protein (RIP1, also called RalBP1 or RLIP76) binds specifically to the GTP-bound form of Ral but not to other GTPases. This protein has GTPase activating activity for Cdc42 and, to a lesser extent, Rac1 but not RhoA, linking the Ral pathway with the regulation of the Rho family proteins (176-178). RIP1 can also bind to Reps1, an Eps-homology domain protein which is tyrosine phosphorylated in response to EGF stimulation of cells and binds to the SH3 domains of the adaptor proteins Crk and Grb2 (179). This chain of proteins may coordinate the cellular actions of activated EGF receptors, Ras, Ral and Rac/Cdc42/Rho proteins.

Although the functions of Ral have long remained elusive, it is known that Ral is required for Src- and Ras-dependent activation of phospholipase D (PLD; 180). Phospholipase D associates directly with RalA, but RalA has no effect upon the activity of PLD. However, PLD is activated when a functional complex PLD-RalA-Arf is formed, where activated Arf is able to activate PLD. Inhibition of the Arf GDP-GTP exchange inhibits PLD activity in Src and Ras transformed cells, implicating Arf in the transduction of intracellular signals activated by Src and mediated by the Ras-RalA cascade. This results show a connection between Ras-RalA and the vesicle formation and trafficking in the Golgi, which is stimulated by both Arf and PLD (181). Other member of the RalGDS family, Rgr, lacks the Ras-binding domain and the ability to bind Ras suggesting a role in the Ras-independent activation of Ral and phospholipase D (182).

RalGDS can also be involved in the activation of gene expression by Ras, since it is able to simulate *fos* expression. RalGDS and Raf act synergistically to stimulate *fos* expression although RalGDS and Rac did not (183). Although Ral seems to be one of the targets of Ras-RalGDS signaling, several observations indicate than RalGDS could signal to other molecules besides Ral. The effects of RalGDS on cellular responses are different from those of active Ral. RalGDS, for instance, can induce low serum and anchorage-independent growth, and tumorigenicity in NIH 3T3 cells, whereas active Ral has no effect (15). In addition, they show different ability to induce *fos* (183) or to cooperate with Raf in cell transformation (171, 174). Although a dominant negative Ral mutant is able to inhibit Ras transformation, this mutant is also able to inhibit cell transformation by Raf, suggesting that this inhibition may be a more general phenomenon (15, 171).

5.3. Phosphatidylinositol 3-Kinase

Phosphatidylinositol 3-kinase (PI3K) is a kinase that catalyzes the phosphorylation of phosphatidylinositol (4,5)-biphosphate (PtdIns[4,5]P_2) to yield phosphatidylinositol (3,4,5)-triphosphate (PtdIns[3,4,5]P_3) in response to many growth factor and cytokines. Several species of PI3K have been cloned and characterized. Heterodimeric PI3Kα and PI3Kβ consist of a p110α or p110β catalytic subunit, respectively, and different p85 adaptor molecules and are

activated by receptors with associated tyrosine kinase activity. A different PI3K isotype, PI3Kγ is activated by the heterotrimeric G proteins and does not interact with the p85 subunits.

Ras has been shown to bind and activate the p110α catalytic subunit of PI3K (184, 185). This interaction requires GTP but not any other protein. The Ras-PI3K binding is blocked by peptides that block Ras-effector binding and was not observed in some of the Ras effector loop mutants (131). The association with PI3K was found in some other GTPases such as Rap1A, Rap1B, R-Ras and TC21, but not in any of the Rho family proteins. Several experiments have shown, furthermore, that Ras controls the activity of PI3K and that the PI3K pathway is responsible for some of the effects of Ras on the cells (131, 184, 185). Both EGF and nerve growth factor (NGF) induced increase of PI(3,4,5)P3, this increase being blocked by dominant negative forms of Ras (Ras17N).

PI3Ks and their lipid products PtdIns(3,4,5)P3 and PtdIns(3,4)P2 act on pathways that control cell proliferation, cell survival and metabolic changes, often through two different protein kinases: protein kinase B (PKB) and p70 ribosomal protein S6 kinases ($p70^{S6K}$). PtdIns(3,4,5)P3 directly stimulates PKB (also named Akt) by binding to the pleckstrin homology (PH) domain of PKB, causing its translocation to the membrane and enabling other kinases to phosphorylate and activate PKB. Four different isoforms of these PKB/$p70^{S6K}$ kinases have been recently isolated (reviewed in Ref. 186). PKB influences metabolism through phosphorylation of glycogen synthase kinase-3 and phosphofructokinase, as well as transmitting a potent survival signal. Thus, Ras has been shown to be able to protect cells from apoptosis through activation of either PKB or NF-κB. (8, 187-189). p70S6K, on the other hand, participates in the translational control of mRNA transcripts containing 5' polypyrimidine tracts. Activation of the physiological levels of PI3K is sufficient to induce transcription from the *fos* promoter and stimulate DNA synthesis in quiescent cells. PI3K mediated DNA synthesis requires both p70 S6 kinase and the Ras-Erk pathway (190, 191).

In vivo, p85 and Ras synergize to induce PI3K activity. Activation of endogenous Ras exerts a small effect on PI3K activity, sufficient for PKB activation, but not lamellipodium formation. However, ectopic expression of activated Ras did result in both PKB activation and lamellipodium formation (192). Thus, other signals are required for full activation of PI3K including one mediated by the regulatory subunits p85. It is likely that Ras activation of p110 synergizes with the interaction of tyrosine phosphoprotein receptors with p85 and both inputs are required to achieve good activation of lipid kinase activity in response to growth factors. The combination of different PI3K catalytic subunits and different p85 regulatory subunits could also link Ras activation of PI3K with other signaling pathways.

The PI3K-PKB route controls signaling pathways regulating different cell processes such as cell survival, glucose uptake, and glycogen metabolism. Some of these processes are also regulated by cell cycle-dependent mechanisms and some of the connections between PI3K and the cell cycle are discussed below. PI3K may also provide a link between Ras and the Rho GTPases and appears to function upstream of Rac, possibly by generating 3' phosphorylated phosphoinositides that activate Rac GEFs (193). Recently, Ras and PI3K activities were found to be necessary for Rac activation through the exchangers Sos and Vav (99, 109). Thus, PI3K seems to be one effector by which Ras induces the cytoskeleton changes dependent on the Rac, Cdc42 and Rho proteins.

5.4. Rho family: Rac, Cdc42 and Ras transformation

Members of the Rho family, the Rho, Rac and Cdc42 GTPases, coordinately regulate the organization of the actin cytoskeleton and the JNK pathway. Rho controls the assembly of actin stress fibers and focal adhesion complexes, Rac regulates actin filament accumulation at the plasma membrane to produce lamellipodia and membrane ruffles, and Cdc42 stimulates the formation of filopodia (194). All of these proteins also stimulate progression through G1 and subsequent DNA synthesis and their inhibitory forms are able to block serum-induced DNA synthesis (195). Two of the members, Rac1 and Cdc42, selectively stimulate the JNK/SAPK cascade leading to subsequent c-Jun transcriptional activity (196, 197). RhoA is also involved in SRF activation and acts synergistically with Rac1 and Cdc42 through a JNK-independent pathway (198).

All the three proteins Rho, Rac and Cdc42 are involved in Ras transformation as demonstrated by different studies with their activated and inhibitory forms. Activated versions of these proteins have a low transforming potential and the produced foci show a different phenotype than those of the Ras-transformed cells; however, these proteins cooperate with Raf in cell transformation and the dominant-negative forms of Rac1, RhoA and RhoB are able to inhibit Ras-induced transformation, indicating an essential role for these proteins in transformation by Ras (199-202). Recently, some of the Rho family members, Cdc42, Rac1 and RhoG, have been shown to cooperate among themselves to transform NIH 3T3 cells independently of Raf. Interestingly, the focus-forming activity elicited by the association of these three proteins uses pathways independent of the Erk and JNK MAP kinases (203). Rac1 and RhoA have been also directly implicated in the morphogenic and mitogenic responses to transformation by oncogenic Ras (204).

The direct link between Ras and the Rho family proteins has remained elusive for several years. Some putative intermediates include a p190 Rho-GAP protein that interacts with p120 Ras-GAP, the Ral binding protein RIP1 that contains a Rho-GAP domain, and PI3K (9, 194). Recently, two mechanisms of Ras-dependent activation of Rac have been reported. Both involve two different Rac exchange factors with Dbl homology (DH) domains regulated by PI3K. Vav is a Rac exchange factor, regulated by Lck-dependent phosphorylation, that binds to and is regulated by the substrate PtdIns(4,5)P2 and the product PtdIns(3,4,5)P3 of PI3K. Lck-dependent activation of Vav is inhibited by PtdIns(4,5)P2 and enhanced by PtdIns(3,4,5)P3 (109). Secondly, the Ras exchange factor Sos contains also a DH domain able to exchange guanine nucleotides on Rac. This activity is

inhibited by other region of the Sos protein, the PH domain. Interestingly, this inhibition is blocked by activated Ras in a PI3K-dependent manner (99, 100). In fact, most Dbl family members contain a DH domain in tandem with a PH domain (104). These PH domains can also regulate the targeting of DH domains to the appropriate subcellular location. In both cases, the Rac activation, dependent on the Ras/PI3K signaling pathway, may initiate the activation of a cascade of GTPases including Cdc42 and Rho. The Rho family of GTPases frequently acts in a GTPase-cascade manner in different cell types (40, 205). In other systems, as it is the case of Swiss 3T3 cells, Cdc42Hs activation was shown to activate Rac1 which in turn activates RhoA (195). The use of dominant interfering alleles placed Rac1 as an intermediate between Ras and MEKK in the signaling cascade to JNK activation (197). Ras activation also led to activation of Rac1 and RhoA, and the Ras-Rac1-Cdc42-SEK-JNK-c-jun cascade has been shown to constitute a key pathway in some cellular processes including stimulation of DNA synthesis in primary cultures of rat hepatocytes (206). The exact molecular mechanism for these GTPase-to-GTPase activation is unknown although several guanine nucleotide exchange factors can be involved (40). Thus, Rac activation of Cdc42 and Rho can be explained by a Rac-GTP-interacting protein, called OST, which acts as a guanine nucleotide exchange factor for Cdc42 and RhoA (207).

How the Rho family members signal to downstream processes depends on a complex network not yet well understood. Rac and Cdc42 are key activators of the JNK and p38/MPK2 cascades, whereas RhoA does not exhibit this activity. To date, more than 10 targets for Rac have been identified (9, 208). Two of them, $p65^{PAK}$ and MLK were reported to be capable of activating the JNK pathway. $p65^{PAK}$ mediates both Rac/Cdc42-dependent activation of JNK and actin reorganization. Expression of a catalytically inactive mutant of $p65^{PAK}$ inhibits Ras transformation of Rat-1 fibroblasts but not of NIH 3T3 cells. This mutant is not able however to inhibit Raf-1 transformation (209). Recently, a new Rac effector named POSH has been identified using the yeast two hybrid system. In COS1 cells, POSH strongly activates the JNK pathway and induces nuclear translocation of NF-κB, whereas the interaction of Rac with $p65^{PAK}$ or MLK does not trigger JNK activation (210). Recently, the use of effector-loop mutants of Rac, Cdc42 and RhoA -similar to those of Ras- is providing some information about the Rho family-effector pathways involved in Ras transformation (211-215). Two families of protein kinases have been described as RhoA effectors, the ROCKs (also known as ROKs) and the PKN-related kinases (also known as the PRK family), in addition to other several proteins of different functions (9, 215). In the case of RhoA, the ability to cooperate with Raf in focus formation correlates with the binding to the effector ROCK-I, a protein involved in actin fiber formation; however, this effector is not involved in the RhoA ability to induce SRF (215). Transformation by Rac1, however, seems to be independent of both cytoskeletal reorganization and SRF induction (212-214).

5.5. MEKK1 and the JNK cascade

MEKK1 was isolated as the mammalian homologue of the *Schizosaccharomyces* byr2 protein kinase, and was named after its ability to phosphorylate MEK *in vitro*. MEKK1 however does not normally has this function *in vivo* but rather functions as a direct activator of the protein kinase JNKK (SEK1/SAPKK1) which in turn activates the stress-activated protein kinases JNK/SAPK (216, 217). The JNKs regulate c-Jun and ATF-2 (CRE-BP1) activity by direct phosphorylation, which results in increased transcription of target genes (218). Ras is in some cases a regulator of the JNK cascade but it is not clear how MEKK is regulated and whether this protein is a direct physiological target for Ras. MEKK1 binds directly to GST-Ras12V in a GTP-dependent manner through its COOH-terminal kinase domain *in vitro* (219). In addition, JNK and Jun itself both interact *in vitro* with Ras in a GTP-dependent manner (220). However, the *in vivo* significance these interactions remains to be determined.

The JNK activity is also stimulated by Raf via an autocrine mechanism (221), providing a crosslink between different effector pathways. Oncogenic activation of c-Raf-1 induces the expression of the heparin binding epidermal growth factor, which putatively activates JNK1 (149, 221).

Recently, both H-Ras and Raf have been shown to activate the transcription factor NF-κB, not through induced nuclear translocation, but rather through the activation of the transcriptional function of the NF-κB RelA/p65 subunit (222). However, inhibitors of the MAP kinase pathway do not block the ability of Ras to activate B-dependent transcription, and dominant negative forms of kinases in the SEK/JNK pathway are indeed able to block this response. Thus, the ability of Raf to activate NF-κB could be explained by the Raf-dependent stimulation of JNK (222).

5.6. GAPs

Several observations have implicated some GAPs, p120-GAP and NF1, as part of the effector system for Ras, in addition to their well-established role as potent negative regulators of Ras (7, 116). These GAPs bind Ras in a GTP-dependent manner and they interact with the Ras effector domain (223), suggesting that GAPs might possibly function as putative targets downstream of Ras in the signaling cascade.

The Ras-GAP catalytic domain of p120GAP and NF1 represents only a small region of the protein sequence and many other functional regions have been identified in these molecules. For instance, p120GAP contains an SH3 domain flanked by two SH2 domains, a pleckstrin homology (PH) domain, a calcium-dependent lipid binding domain, a stretch of prolines (type I or II) and a site of interaction with phospholipids. These domains mediate interactions with many different proteins including p190 (Rho-GAP), EGFR, Src, Lck and Bcr-Abl (116).

Some functional experiments suggesting an effector role for GAPs are the following:

5.6.1. p120GAP

1. Ras-p120GAP complex was found to inhibit muscarinic receptor-activated potassium channel opening and this effect was shown to be dependent on the SH2-SH3 region of p120GAP (224, 225).

2. Microinjection of an antibody against the SH3 region of p120GAP abolishes H-Ras-induced germinal vesicle breakdown and blocks activation of the kinase cdc2 stimulated by oncogenic Ras in *Xenopus* oocytes (226-228).
3. A p120GAP mutant, lacking the Ras binding domain, and targeted to the membrane inhibited the activity of oncogenic Ras without affecting the action of normal Ras in a focus formation assay (229).
4. p120GAP interacts with a p190 protein and the complex p120GAP-p190 induces the GTPase activating function of Rho proteins, correlating with disruption of the actin stress fiber network and cell adhesion, probably resulting from a deregulation of the Rho and Rac GTPases (230). Accordingly, overexpression of p120GAP impairs the chemotactic response of fibroblasts to PDGF and lysophosphatidic acid (231). Since these processes are not abolished in dominant negative Ras-expressing cells, it is not clear if Ras-p120GAP association plays a role in these processes (232).
4. Dominant negative Ras, however, does inhibit the binding of p120GAP with G3BP, a protein showing similarity to RNA binding proteins and which has been proposed to affect both the stability and translation efficiency of mRNAs (116, 233).

5.6.2. NF1

1. Overexpression of neurofibromin results in severe growth inhibition without an effect on Ras-GTP levels in NIH 3T3 cells (234).
2. Neurofibromin or its Ras GTPase domain is able to inhibit the transforming or proliferative effect of the *ras* oncogene, despite its inability to stimulate the GTPase activity of oncogenic Ras (42, 112, 235). For instance, expression of the GAP-related domain of NF1 in a $cdc25^-$ yeast strain inhibits growth supported by the H-ras proto-oncogene as well as by the H-Ras oncogene (236), and NF1 is able to suppress tumorigenicity of the HCT116 human colon carcinoma cell line, which harbors an oncogenic Ki-*ras*, in nude mice (237). Moreover, the transformation of NIH 3T3 cells by oncogenic *ras* is inhibited by overexpressed full-length neurofibromin (Johnson et al. 1994).
3. The Ras GTPase domain of NF1 is able to inhibit H-*ras* oncogene-induced anchorage independent growth in NIH 3T3 cells (238).
4. Most of the mutations found in NF1 patients leave the GRD domain intact (239-241), suggesting that alterations of functions other than the GTPase accelerating activity of NF1 may be associated with tumorigenesis. In addition, a GAP-related domain peptide of NF1 carrying a mutation that confers greatly diminished GAP activity, but a normal binding affinity for Ras-GTP, suppresses tumor formation by the HCT116 human colon carcinoma cell line (237).
5. NF1 deficiency cooperates with N-Ras protooncogene overexpression in the activation of Erks and the induction of tumors in transgenic mice. However, this cooperation is independent of the GTPase activity of NF1 since Ras-GTP levels are not altered by the NF1 deficiency. These results could suggest a role for NF1 in inhibiting the activation of the Erk pathway by competing with Raf for Ras (242).

5.7. Other putative Ras effectors: PKCζ, AF6, Canoe, Rin1 and Nore1

Other putative Ras effectors have been characterized by their binding to the GTP-bound form of Ras, but whose function is not well understood. PKCζ is a serine/threonine PKC atypical isoform that is insensitive to diacylglycerol and calcium. The direct regulation of PKCζ by Ras was uncovered by the analysis of the association between Ras-GTP and the N-terminus of PKCζ and coprecipitation studies, suggesting a role for this PKC isoform in Ras signal transduction (243). In addition, the dominant negative form of Ras is able to inhibit PKCζ activity in growth factor-stimulated NIH 3T3 cells. Ras could serve to localize PKCζ to the plasma membrane allowing its activation by phosphatidylinositol derivatives. PKCζ could also be involved in the Ras-dependent protection of some cells from apoptosis (244).

AF-6 and Canoe are two related proteins found to bind to GTP-Ras. Their function is unknown but they contain motifs involved in specific cell-cell interaction. AF-6 is also found as the fusion partner in acute lymphoblastic and myelocytic leukemia translocations (245). Rin1, for Ras Interaction/Interference, has also been shown to directly associate with Ras in a GTP and effector loop-dependent manner *in vitro* and *in vivo* (246). This protein is located in discrete patches in the plasma membrane and its function is unknown. Recently, a new putative Ras effector named Nore1 has been isolated in a yeast two-hybrid system (247). This protein has no significant sequence similarity to any other mammalian protein but is homologous to a *Caenorhabditis elegans* gene product called T24F1.3, which has been suggested previously to contain a Ras/Rap association domain (248). Nore1 lacks an identifiable catalytic domain but contains a cysteine-histidine-rich element typical of a diacylglycerol/phorbol ester binding site and several putative SH3 domain binding sites. Importantly, Nore1 has been demonstrated to associate with Ras *in vivo* following EGF receptor and PMA activation in COS-7 cells and in the human oral carcinoma cell line KB (247). Thus, although the function of this protein is still unknown, Nore1 seems to be a true Ras effector that will increase the complexity of the Ras-dependent cellular networks.

Ras-binding (RA) domains have been found in other proteins through motif searches in the databases. RA domain-containing proteins include Rgl, Rlf, RalGDS, AF-6, Canoe, Rin1 (known to bind one or more Ras isoforms) and other proteins such as myr-5, diacylglycerol kinases, class IX myosins and a phospholipase C β-homologue. (248). The presence of other domains present in these proteins suggest putative functions for these interactions. Thus, myr-5, which also contains a Rho/Rac GTPase-activating sequence, could link Ras to Rho inactivation, following the pattern of other GTPase cascades (205).

6. Ras AND THE CELL CYCLE CONTROL

Cell cycle control is a complex process by which cells decide whether to proliferate or to stay quiescent. Many different proteins are involved in this process. Basically, the engine that produces cell cycle progression is represented by a family of protein kinases, the Cyclin-Dependent Kinases (CDK). The activity of these molecules is dependent on the presence of a stimulating subunit, the cyclins, named after their tight regulation through the cell cycle phases, and an inhibitory subunit, the CDK inhibitors (CKIs). In addition,

other several kinases and phosphatases are also involved in the regulation of these molecules (249, 250).

Early studies demonstrated that Ras induces DNA synthesis in the nucleus of quiescent cells (251), suggesting that the Ras signaling pathway is linked directly to the G1/S phase transition of the cell cycle. In fact, the only phase when inhibition of Ras affects cell cycle progression is G1 (251). Once cells have entered S phase, Ras become dispensable until the next cell cycle.

In general, Ras plays an important role integrating mitogenic signals with the cell cycle progression. Ras mediated signals induce gene expression, as demonstrated by studies on the early-responsive transcription factors. Transient expression of oncogenic Ras induces expression of endogenous Jun and Fos, activating the AP-1 transcription factor complex (7). In addition, other transcription factors such as the PEA-3 element and the members of the ets family are also activated by Ras.

The molecular basis of cell cycle control and the regulatory role of CDKs, cyclins and CKIs has been extensively studied in the last few years. The understanding of these processes has led to the analysis of the role of cell cycle regulators in Ras signaling. All this work has provided information about how Ras produces so many different responses in cells, such as cell proliferation or cell cycle arrest, and how different genetic alterations cooperate in cellular tumorigenesis.

6.1. Cyclin D-CDK4/6 complexes and the Retinoblastoma pathway

Few cyclin-CDK substrates have been unequivocally identified to date. One of the most important for G1 progression is the product of the Retinoblastoma gene (Rb). Regulation of the phosphorylation state of the retinoblastoma protein is a key event in the progression of cells from G1 phase into S phase. In growth-arrested or early G1 cells, Rb is hypophosphorylated and binds to transcription factors of the E2F family, recruiting histone deacetylase (HDAC) and thus repressing transcription (252-254). Cyclin D1 is synthesized during G1, binding and activating CDK4/6, which phosphorylate Rb at multiple sites. Hyperphosphorylated Rb does not longer bind to the E2F family of transcription factors leading to the dissolution of the E2F-Rb-HDAC complexes and allowing gene transcription and the progression of the cell cycle to the DNA synthesis phase (S). Different groups have reported the induction of expression of cyclin D by Ras (255-262). The shortening of the G1 phase, detected in Ras transformed cells, can be associated to an increased expression of cyclin D1 and, in fact, can be abrogated by a cyclin D1 antisense. However, although constitutive overexpression of cyclin D1 accelerates G1 progression, cells remain untransformed, indicating that cyclin D1 may be necessary but it is not sufficient for the transforming activity of Ras (257, 260). Cyclin D1 activation by Ras seems to be dependent on the Raf/MAP kinase pathway (263, 264) and the AP-1 like sequences present in the cyclin D1 promoter are involved in this activation (256).

Cyclin D induction by Ras has been shown to be necessary for Ras-induced anchorage-independent growth (257). Others have shown that Ras also up-regulates cyclin A, D3, and E, and the E2F family of transcription factors (262). Ras-dependent Cyclin A induction seems to be specially important for anchorage-independent growth, at least in some cell types (265).

Inactivation of Ras by the use of dominant negative forms in cycling cells causes a decline in cyclin D1 protein levels, accumulation of the hypophosphorylated, growth-suppressive from of Rb, and G1 arrest (266, 267). This G1 arrest can be corrected by forcing the expression of cyclin D1. Interestingly, when Rb is disrupted, cells fail to arrest in G1 following Ras inactivation (266). When a neutralizing antibody directed against Ras was microinjected, cells without Rb or $p16^{INK4a}$ were more resistant -although not completely- to the inhibitory effects of the anti-Ras antibody, indicating that Ras is required for Rb inactivation but has also other functions in cell-cycle progression (268). Thus, the ability to induce Rb phosphorylation through cyclin D-dependent kinases, although does not account for all aspects of Ras regulation of the cell cycle, seems to be a major part of the link between Ras and the cell-cycle machinery (269).

6.2. Cyclin E-CDK2 complexes and the $p27^{Kip1}$ inhibitor

Cyclin D, while necessary, is not sufficient for G1 progression. Cyclin E, which protein peaks at the G1/S phase border, binds to CDK2, and is also required for G1-S progression. Thus, Ras signals should also activate these cyclin E-CDK2 complexes to enter in cell proliferation. Some reports indicate that Raf weakly induces the expression of cyclin E and cyclin A (258, 270); however, the activation of the cyclin E-CDK2 complexes is mainly achieved through the degradation of the cell cycle inhibitor $p27^{Kip1}$ (260-262, 267, 271). Conditional expression of oncogenic Ras induced the G1-S transition in rat fibroblasts and significantly reduced the synthesis and shortened the half-life of $p27^{Kip1}$ protein (261, 271). Although the $p27^{Kip1}$ protein levels are tightly regulated *via* the ubiquitin pathway (272), the Ras-dependent protein degradation was found to occur in a proteosome-independent way (261). The lack of $p27^{Kip1}$ degradation can be sufficient to stop Ras-induced proliferation; thus, in BALB c/3T3 cells, but not in NIH 3T3, expression of activated Ras failed to increase the cyclin dependent kinase activity on Rb, because of the constant presence of the CDK inhibitor $p27^{Kip1}$ (258).

Several reports indicate that Ras causes induction of $p27^{Kip1}$ degradation through the MAP kinase signaling pathway (260, 261, 267, 271). This degradation is dependent on the MEK/MAP kinase activities as shown by the use of cyclic AMP-elevating agents and a MEK inhibitor (261). Furthermore, $p27^{Kip1}$ has been shown to be phosphorylated directly by MAP kinase *in vitro* and the phosphorylated protein cannot bind to and inhibit CDK2 (261). The specific activation of the MEK1-Erk pathway, however, is not sufficient to trigger degradation of $p27^{Kip1}$ (264). The Raf-MEK-Erk pathway is involved in the induction of cyclin D and the levels of cyclin D-CDK complexes could sequester $p27^{Kip1}$. Thus, in cells overexpressing cyclin D1 and CDK4 subunits, these complexes have been reported to sequester $p27^{Kip1}$ and reduce its effective inhibitory threshold. Therefore, the MEK-Erk pathway could function

posttranslationally to regulate cyclin D1 assembly with CDK4 and thereby to help cancel $p27^{Kip1}$-mediated inhibition (264).

6.3. Senescence response to Ras signals ($p16^{INK4a}$, $p15^{INK4b}$, p53 and $p21^{Cip1}$)

Recently, several studies have found an apparently contradictory function of activated Ras proteins. Expression of Ras in primary cells is able to strongly induce expression of cell cycle inhibitory molecules such as p53 and $p16^{INK4a}$ (273). Activated Ras up-regulated p53, correlating with enhanced p53 transactivation and up-regulation of $p21^{Cip1}$ and Gadd 45, two p53 effectors and negative cell regulators (262). This activated Ras-dependent response causes an arrest in the cell cycle progression of rodent and human primary fibroblasts and shows characteristics indistinguishable from cellular senescence. Cells develop a flat morphology and express specific markers of senescence (273). Cell transformation by Ras is hence inhibited since forcing the expression of high levels of the CDK inhibitors $p16^{INK4a}$ (274) and $p21^{Cip1}$ (275) blocks the ability of oncogenic Ras to cause proliferation and transformation in murine fibroblast cell lines. In primary rat Schwann cells, however, Ras-specific induction of the p53-dependent inhibitor $p21^{Cip1}$ results in cells that retain certain properties of transformation -refractile morphology and increased motility- but growth arrested in the G1 phase of the cell cycle (276).

Activated Raf has also been shown to lead to a G1-specific cell cycle arrest through induction of $p21^{Cip1}$, inhibition of cyclin D- and cyclin E-dependent kinases and an accumulation of hypophosphorylated Rb (277). Importantly, only very high levels of activated Raf are able to induce this response, whereas a less strong Raf signal induces cyclin D1 and the progression of the cell cycle (270, 278). These results are in concordance with previous work with a series of activated Raf molecules showing that Raf proteins able to induce proliferation activate Erk kinases only weakly, whereas Raf molecules strongly activating Erks inhibit proliferation (279).

Although activation of the Raf-Erk pathway leads to the induction, in a p53-dependent manner, of the CKI $p21^{Cip1}$ (276, 277), a more complex signaling seems to be required for the induction of $p16^{INK4a}$. Multiple Ras downstream pathways are required for the induction not only of $p16^{INK4a}$ but also of the cell cycle inhibitor $p15^{INK4b}$, a member of the INK family involved in G1 control and able to inhibit cell transformation by Ras (M. M. & A. P., submitted). Both $p16^{INK4a}$ and $p15^{INK4b}$ are likely to cooperate in the senescence response to activated Ras signals. As discussed in the next heading, Ras may need the cooperation of other molecules, mainly nuclear oncogenes, to overcome the inhibitory effect of these cell cycle inhibitors.

6.4. Cooperation between oncogenes to overdrive the cell cycle

Carcinogenesis is clearly a multistep process and to reach the malignant phenotype requires multiple alterations affecting several levels of growth control. In contrast to established lines, primary cells are not usually susceptible to full transformation by mutant *ras* alone. Early gene transfer experiments demonstrated that two or more cooperating oncogenes are required to convert normal cells to a tumorigenic state (280-282). Transformation of primary fibroblasts by *ras* requires cooperation with immortalizing oncogenes such as c-*myc*, N-*myc*, E1A, or polyoma large T. These observations indicated that *ras* oncogenes can drive transformation but they are highly inefficient in overcoming senescence. However, establishment as continuous cell lines is not sufficient for transformation by *ras* (283) and, for instance, transformation of rat REF52 cells with *ras* oncogenes requires complementation with E1A or SV40 (284). This resistance of primary cells to be transformed by Ras is now explained by the effect of strong Ras signaling on the cell cycle inhibitors (see above). The ability of oncogenic Ras to induce the expression of the CKIs is thought to be a protective or stress response of the cell when receiving a Ras signal at an inappropriate stage in the cell cycle (269, 285).

This protective response, therefore, has to be overcome by cooperating oncogenes in order to transform cells. This effect can be obtained either by the loss of function of the inhibitory proteins or through the expression of cooperating molecules. Thus, in primary rat Schwann cells, the attenuation of $p21^{Cip1}$ induction, either by antisense expression or by inhibiting p53 activity, results in a loss of the growth inhibitory signal from Ras (276). Cooperation between loss of function of many of the CKIs and Ras has been studied *in vivo* in knockout mice. Primary cells from p16-, p21-, or p53-deficient mice can be transformed by Ras alone (273, 286).

Since the abrogation of the negative growth signal from Ras is sufficient to unleash its transforming activity, many of the proteins that cooperate with Ras in primary embryonic fibroblast transformation have the ability to counteract the cell-cycle inhibitory effects of CKIs (285). For instance, overexpression of cyclin D, cyclin E, or cdc25 cooperates with Ras in rat embryonic fibroblasts (REF) transformation presumably by increasing the activity of cyclin/CDK complexes over the threshold imposed by the CKIs (287-289). Deregulation of the E2F transcription factors, which act downstream of Retinoblastoma, also leads to transformation of rat embryo fibroblasts in cooperation with Ras and E2F1 itself induces anchorage-independent growth in immortalized 3T3 or REF cells (290). Viral oncogenes cooperate with Ras by blocking either the effects of p53 (human papilloma E6, E1A, SV40LT), $p16^{INK4a}$ by binding Rb (human papilloma E7, SV40LT) or $p21^{Cip1}$ (E7) (285).

The 'classic' transforming cooperation between Ras and Myc has been widely analyzed *in vitro* (291) and *in vivo* (292), and, recently, this cooperation is being understood as a result of the effect of these proteins on the cell cycle regulators. Whereas Ras induces cell cycle progression activating cyclin D proteins, Myc is essentially a positive regulator of G1-specific CDKs and, in particular, of cyclin E/CDK2 complexes. Myc acts *via* at least three distinct pathways which can enhance CDK function: (1) functional inactivation of the CDK inhibitor $p27^{Kip1}$, (2) induction of the CDK-activating phosphatase Cdc25A and (3) deregulation of cyclin E expression (293). Thus, Ras and Myc collaborate in

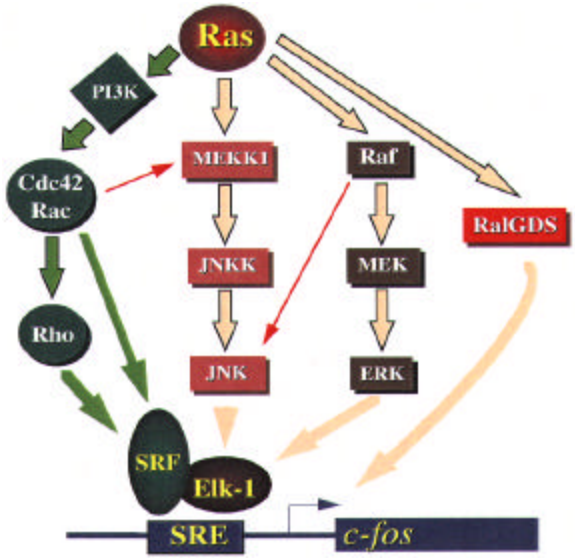

Figure 3. Ras mediates control of c-*fos* expression through different pathways. Rac/Cdc42 and Rho proteins activate the serum response factor (SRF), whereas the JNK and Erk kinases transactivate Elk-1 (TCF). RalGDS family proteins also activate c-*fos* expression by a mechanism which is not clear.

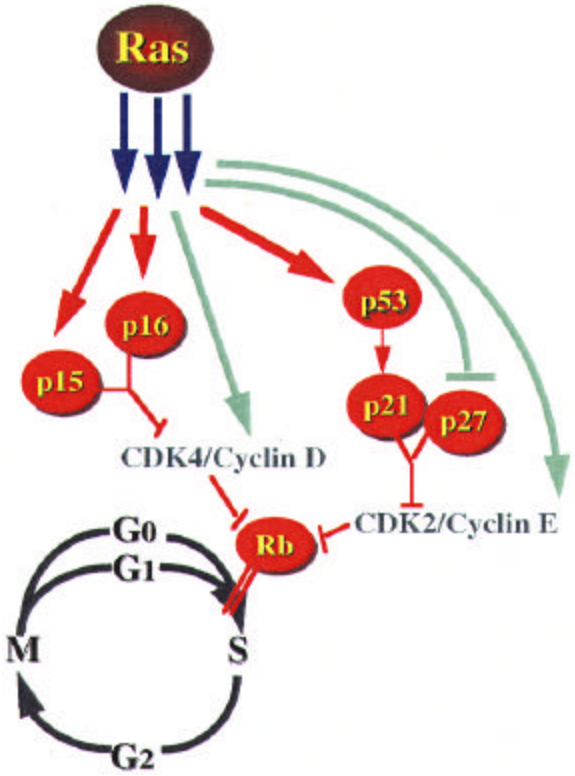

Figure 4. Regulation of the cell cycle by Ras. Ras can participate in cell cycle regulation sending activating (green lines) or inhibitory (red lines) signals through several downstream pathways, depending on the cell context. Activation of the G1->S progression is mediated by induction of cyclin expression and by triggering degradation of the $p27^{Kip1}$ inhibitor. Ras can also induce the CDK inhibitors $p16^{INK4a}$, $p15^{INK4b}$ and $p21^{Cip1}$ producing G1 arrest.

several different points on the cell cycle regulators. All these activities produce a synergistic enhancement of cyclin E/CDK2 and E2F activity (267).

7. PERSPECTIVE

7.1. Multiple pathways to control transcription, morphology and other cellular processes

Ras proteins act at a central point of many signal transduction pathways from extracellular signals to the cytoplasm and the nucleus. Ras activation results in two main cellular changes: reorganization of cytoskeleton and mitogenesis. The analysis of these cellular processes using different effector loop mutants of Ras (129, 131, 204) and the study of different Ras effectors has allowed the dissection of the different pathways downstream of Ras. Although the Raf-Erk pathway has been largely thought to be the main pathway for Ras response, the use of these mutants showed that at least another pathway is responsible for the morphological changes associated with the actin cytoskeleton and is also required, together with the Raf-Erk pathway, for a complete mitogenesis response (204). This pathway is dependent on the Rho family proteins and is frequently mediated by PI3K regulating polymerization of actin, stress fiber formation and focal adhesion (9, 131, 194, 294). Using different downstream pathways, Ras proteins are involved in many cellular processes including transcription, translation, control of cytoskeleton, vesicle formation, cell-cell junctions, etc. Several of these pathways converge in the regulation of transcription.

Transcription of the early-response gene c-*fos* is regulated by different promoter elements. Among these, the serum response element (SRE) is believed to play a central regulatory role (295) (figure 3). Induction through the SRE requires binding of two cellular proteins, the serum response factor ($p67^{SRF}$) and one from a family of proteins able to form a ternary complex with SRE and SRF, therefore termed ternary complex factors (TCF: Elk-1, SAP-1). The ets-domain transcription factor Elk-1 is a substrate for three distinct classes of MAP kinases. Elk-1 has been shown to regulate SRE activity in response to the activation of the Ras-Raf-Erk pathway (144). Furthermore, Elk-1 can also be phosphorylated by JNK (296, 297) and the p38 MAP kinases (146, 298).

In addition, SRE can also be regulated in a TCF-independent manner through members of the Rho family (198). Thus, functional RhoA is required for LPA-, serum-induced transcriptional activation by SRF and Rac1 and Cdc42 also potentiate SRF activity (198). The ability of RhoA and Rac1 to cooperate with Raf in focus formation does not depend, however, on the their activation of SRF, but rather, in the case of RhoA, on the activation of ROCK-I, a RhoA effector involved in the formation of actin stress fibers (214, 215). The pathways or effectors involved in Rho-dependent SRF activation have not yet been elucidated.

7.2. Multiple pathways to control cell cycle regulation

Ras proteins play a key role in integrating mitogenic signals with cell cycle progression through G1. It can be concluded that Ras plays temporally distinct, phase-

specific roles throughout the cell cycle, and particularly in the G1 phase (271). Ras-dependent inputs can be activating or inhibitory signals that reach the cell cycle regulatory machinery using different downstream pathways (figure 4). Ras is required for cell cycle progression and activation of both CDK2 and CDK4 complexes until 2 h before the G1/S transition, corresponding to the restriction point. This is done basically through a Raf/MEK/Erk-dependent induction of cyclin D1 and downregulation of $p27^{Kip1}$. However, although Ras-dependent cyclin D1 induction is only dependent on the Erk pathway, degradation of $p27^{Kip1}$ seems to require additional events. The effect of the Ras/Raf/Erk pathway on cyclin expression may produce an increase in the level of cyclin D/CDK complexes which are able to sequester $p27^{Kip1}$ canceling its inhibitory effect (264). In addition, a RhoA-associated pathway, that could involve a PI3K-dependent but PKB-independent pathway, has been proposed to cooperate in the Ras-dependent $p27^{Kip1}$ downregulation (299, 300). Since dominant negative forms of Erk inhibit $p27^{Kip1}$ degradation, the Erk pathway seems to be necessary but not sufficient for this activity. The PI3K pathway has been shown to synergize with the Raf pathway in inducing DNA synthesis and loss of contact inhibition (131, 204). Ras signaling to the cell cycle machinery also occurs via multiple pathways to induce anchorage-independent growth. Thus, activation of any of the three possible pairwise combinations between the Raf, RalGDS and PI3K pathways is needed in NIH 3T3 cells to induce anchorage-independent growth. Each individual pathway only partially relieves anchorage dependence of Rb phosphorylation, cyclin E/CDK activity and expression of cyclin A (301). In T cells, induction of the E2F transcription factors after IL-2 stimulation has been demonstrated to be dependent on the PI3K/PKB pathway (300). Also, multiple Ras downstream pathways, involving Erk and Rac, have been shown to contribute to regulate the nuclear factor of activated T cells (NFAT) (302).

Recently, the Ras activation changes during the cell cycle have been analyzed. An elegant study demonstrated that in HeLa cells and NIH 3T3 fibroblasts, the increase of Ras-GTP loading achieved immediately after release from mitosis is much less than a second phase of Ras activation that occurred some 5 h latter, in mid-G1 (303). Interestingly, only the first phase of Ras activation was accompanied by Erk activation, whereas the latter, much stronger Ras activation occurred without significant Erk activation. The biological significance of Ras activation in mid-G1 phase, and the nature of the effectors recruited by activated Ras at that time is entirely unknown. Ras activity late in G1 phase is required for $p27^{Kip1}$ downregulation, resulting in activation of cyclin D/CDK4 and cyclin E/CDK2 and entry into S phase (271).

Members of the INK4 and Cip/Kip family often work in concert in response to antimitogenic signals such as TGFβ. Thus, diverse evidence has led to the hypothesis that CKIs establish an inhibitory threshold which must be surpassed in order for the cell cycle to proceed (250). As reported in the few last years, Ras uses multiple pathways to induce the cell cycle activators and to overcome the inhibitory activity of the CKIs. For cells to reenter the cell cycle, they require first to induce cyclin D so cyclin D-CDK4/6 complexes can exceed the threshold established by CKIs and allow activation of cyclin E-CDK2 complexes needed for downstream events. Once this task is accomplished, cyclin D is downregulated by proteolysis and perhaps upregulation of $p16^{INK4a}$ (304). Since Ras signals early and late in the G1 phase, and, on the other hand, Ras is also able to induce $p16^{INK4a}$ and $p15^{INK4b}$ expression through specific sequences in their promoters (273; M. M. & A. P., submitted), it should be interesting to analyze the effect of Ras signaling late in the G1 phase. However, in the event of inappropriate cell proliferation induced by Ras oncogenic signals, the levels of several CKIs, including $p16^{INK4a}$, $p15^{INK4b}$ and $p21^{Cip1}$, are increased providing a way to control the G1 timing and to prevent excessive cell proliferation and subsequent tumorigenesis.

Ras proteins, in summary, are helping us to understand how the cell works as a whole, integrating different signaling cascades. The growing number of Ras regulators and effectors is exponentially increasing the number of cellular processes where Ras activation plays a role, from kinase cascades to cell cycle progression, apoptosis or cell-to-cell interactions. All these cellular proteins and the interactions among them form a complex web that responds coordinately when an external signal reaches the cell. Probably, the web has different organization depending on the cell type, but Ras is frequently in the middle of it.

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