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REVIEW ARTICLES

Ras Family Genes: An Interesting Link Between Cell Cycle and Cancer

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Ras genes are evolutionary conserved and codify for a monomeric G protein binding GTP (active form) or GDP (inactive form). The ras genes are ubiquitously expressed although mRNA analysis suggests different level expression in tissue. Mutations in each ras gene frequently were found in different tumors, suggesting their involvement in the development of specific neoplasia. These mutations lead to a constitutive active and potentially oncogenic protein that could cause a deregulation of cell cycle. Ras protein moderates cellular responses at several mitogens and/or differentiation factors and at external stimuli. These stimuli activate a series of signal transduction pathways that either can be independent or interconnected at different points. Recent observations begin to clarify the complex relationship between Ras activation, apoptosis, and cellular proliferation. A greater understanding of these processes would help to identify the factors directly responsible for cell cycle deregulation in several tumors, moreover it would help the design of specific therapeutic strategies, for the control on the proliferation of neoplastic cells. We summarize here current knowledge of ras genes family: structural and functional characteristics of Ras proteins and their links with cell cycle and cancer. J. Cell. Physiol. 192: 125–130, 2002.

RAS FAMILY

A mammalian cell contains at least three distinct ras proto-oncogenes: H-ras, K-ras, and N-ras (Lowy and Willumsen, 1993). H-Ras and K-Ras were first identified as viral (v-Ras) oncoproteins of Harvey and Kirsten murine sarcoma viruses, and were found to be capable of cellular transformation. The N-Ras oncoprotein was identified in a neuroblastoma cell line. The human genomic DNA sequences span 3 kb (H-ras), 7 kb (N-ras), and more than 35 kb (K-ras) and are located in chromosomes 11p15.5, 12p12.1, 1p13, respectively. The K-ras gene is alternatively spliced into two isoforms: K-rasA and K-rasB (Pells et al., 1997). Other members of the Ras family genes are M-Ras, R-Ras, Rap 1/2, and Ral that share at least 50% sequence identity. Several monomeric G protein family members (Rho/Rac/Cdc42, Rad, Ran, Arf, Rab/Ypt) share at least 30% homology with Ras family genes (Wittinghofer and Herrmann, 1995). Rap 1 is involved in a number of cellular processes such as T-cell anergy and platelet activation while the function of Rap 2 is still unclear.

H-, K-, and N-ras have similar structure and sequences, with five exons, first of which not codifying, and conserved splicing sites, even if the introns have various dimensions and sequences (Lowy and Willumsen, 1993). The promoters of these genes are TATA-less and have upstream GC elements regulating their expression. The main product of the ras gene is a monomeric G protein of 21 kDa which is able to bind and hydrolize guanosine triphosphate (GTP). Ras is ubiquitously expressed although mRNA analysis suggests different tissue expression levels. H-ras is highly expressed in the skin and in skeletal muscles, K-ras is mostly expressed in the colon and in the thymus, and N-ras in male germinal tissue and the thymus suggesting that ras family members probably are expressed in a tissue specificity fashion (Lowy and Willumsen, 1993). In support of this hypothesis, mutations in each one of these genes,

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frequently found in various types of tumors, are involved in the development of specific neoplasia, such as mutations of K-ras in lung, colorectal and pancreas tumors, H-ras in bladder, kidney and thyroid tumors, and N-ras in melanoma, hepatocellular carcinoma, and hematological malignancies (Fujita et al., 1984; Visvanathan et al., 1988; Bos et al., 1987; Bos et al., 1989; Hesketh, 1995). Mutations found in the tumors always lead to the expression of constitutively active protein product.

RAS PROTEINS

The three main Ras proteins share high homology in the first 165 amino acids but show difference in 25 amino acids of the carboxyl-terminal region that constitutes the heterogenous region. All the Ras proteins contain a terminal CAAX box in the 186–189 position. In this box, the "C" represents cysteine, "A" represents an aliphatic amino acid (leucine, isoleucine, or valine), and "X" is metionine, serine, leucine, or glutamine. The post-translation modifications, beginning from amino acid 186, (always cysteine), increase the hydrophobicity of the carboxyl-terminal region of the protein allowing its inner in plasma membrane. The membrane localization of these proteins is essential for their function. In fact, mutations in region 186–189 determine the cytosolic protein, inactivate Ras, and suppress its transforming activity. The post-translation modifications are the farnesylation of C-186 by the cleavage of the three downstream amino acids (AAX), followed by methylation of C-186 and, finally, a palmitoylation of cysteine residuals in the region 165–186 (Clarke, 1992; Hesketh, 1995). Cysteine mutation in the CAAX box prevents farnesylation and Ras function.

Structural characteristics of Ras proteins

The three-dimensional structure achieved by a protein at the end of its folding process represents a thermodynamically more stable conformation. Generally, this conformation depends on its amino acidic sequence, or at least on those from its conserved regions that cannot be substituted because they carry the most important information for determining the correct three-dimensional protein structure. The proteins are flexible and are capable of conformational fluctuations. This structural mobility has important functional relevance. The mutations of conserved regions are responsible for the functional protein alteration.

Five uncontiguous domains (5–63, 77–92, 109–123, 139–165, and 186–189) are essential for Ras activity, and mutations in these regions block the transforming ability of oncogenic protein (Bos, 1989).

Point mutations in 12, 13, 59, 61 codons block the GTPase activity, leading to constitutively active and potentially oncogenic protein. The mutations in 186 codon inactivate Ras blocking its membrane insertion where its regulators and effectors are localized.

X-ray analysis of Ras in complex with GTP or GDP equally provides an atomic description of the native protein and the oncogenic form (De Vos et al., 1988; Krengel et al., 1990), thus indicating that these amino acids are important for protein function. The p21-Ras is essentially constituted of six beta sheets and five alpha helixes, connected by ten loops (Bos, 1989), (Fig. 1). Loop L1 contains glycin residues, Gly12 and Gly13, which are most frequently mutated in human tumors, and lead to constitutive active protein. The most important residues for the interactions of p21Ras with their effectors (32–40 position) are localized to loop L2. Loop L4 contains glutammine 61 (Gln61) whose substitution has an oncogenic effect. The residues involved in GTPase activating protein binding GAP/NF, are in position 30–38 which also is the domain of Ras interaction with its effectors (Bos, 1989). The amino acids in position 12, 59, and 61 are essential for Ras GTPase activity by GAP/NF stimulation. In particular, the Gln61 stabilizes the external GTP phosphoric group and carries one H_2O molecule essential for nucleophile attacking. Mutations in 12 and 59 codons interfere with the corrected Gln61positioning in the transition complex during GTP hydrolysis. GAP/NF contributes to Gln61 positioning in the Ras catalytic site formation by a conserved arginine residue in all GAPs. This residue interacts with the nucleotide phosphates and neutralizes the negative charge. Thus, mutations in Gly12 and Gln61, inhibit GTP hydrolysis. Mutations in Ala59, reduce the exchanged nucleotide amount, block Ras in the complex binding GTP, and lead to constitutively active protein (Scheffzek et al., 1998).

Ras becomes activated during physiological conditions as a result of extracellular signals, by the effect of its interaction with guanine exchange factors (GEFs). Crystallographic analysis of Ras in complex with SOS (one of the best known GEF) reveals that GEF stimulates GDP release. SOS induces a Ras conformational change that opens the nucleotide-binding site, and blocks the interaction with GDP phosphates and Mg^{++} (Boriack-Sjodin et al., 1998; Scheffzek et al., 1998).

SOS dissociates the nucleotide from Ras in < 1 sec. In the absence of SOS, this nucleotide is released with a K_d of 10^{-5} sec, meaning only after a couple of hours. In addition, the Ras/SOS complex is not too stable. The binding site for the nucleotide remains accessible and GTP molecule, particularly abundant in the cell, shifts SOS to bind Ras (Boriack-Sjodin et al., 1998; Wittinghofer, 1998). Ras/GTP complex does not remain active for a long time but the fine control operated by GAPs inactivates Ras by stimulating GTP hydrolysis.

Ras functional properties

Ras protein moderates cellular responses at several mitogens and/or differentiation factors (i.e., growth factors, cytokines, cellular adhesion signals) and at external stimuli, such as irradiations UV, osmotic stresses, among others (Downward, 1998; Hodgkin et al., 1998; Gille and Downward, 1999; Kresse and Schönherr, 2001).

These stimuli activate a series of signal transduction pathways that can be either independent or interconnected at different points Ras is activated after interaction of several growth factors (EGF, PDGF, etc.) with their tyrosine kinases receptors that, interacting with their ligands, autophosphorylate in tyrosine (Downward, 1998; Gille and Downward, 1999).

Adaptor proteins, like growth factor receptor-bound protein 2 (GRB2), interact with receptor phosphorylated tyrosines by their SH2 domains and bind Ras-GEF by the SH3 domain. GEFs are translocated in the

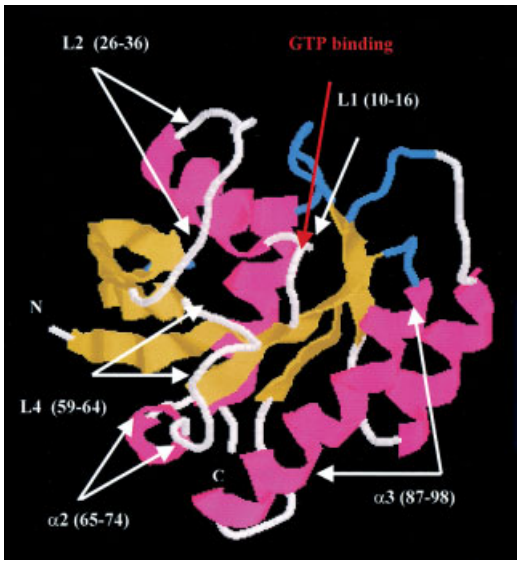

Fig. 1. Three-dimensional structure of Ras protein. This figure was elaborated using RasMac 2.6 and the entry #521P from the Protein Data Bank (Brookhaven National Laboratory).

membrane where they promote the RAS transition into a Ras-GTP active complex. Ras is quickly inactivated by GAPs that stimulate GTP hydrolysis. Although transitory, Ras active protein is sufficient to stimulate cell signal transduction. The timing and quantity of Ras activation depends on the intensity of the primary signal and also on its specific nature (Wan et al., 1996) Ras activation could lead to different signal transduction pathways responsible for different cellular responses. Serpentine receptors (STMR), interacting with trimeric G proteins, also can induce Ras activation (Wan et al., 1996).

The three main Ras effectors (Fig. 2), RAF kinase, RAL-GEFs, and PI3-K, bind the same region of Ras-GTP, the 32-40 domain. All three effectors increase their "in vivo" activity after the Ras binding. The best known Ras stimulated pathway starts with activation of the serine-threonine RAF. Recent studies suggest that Ras interacts with the amino terminal portion of RAF located in the cytoplasm and complexed with 14-3-3 protein which is an esential cofactor of Raf kinase activity (Tzivion et al., 1998). This interaction causes a RAF conformational change unmasking one or more residues of phosphorylation and stabilizing a new catalytic active RAF conformation. After these conformational changes, RAF is anchored in plasma membrane by a partially clear mechanism (Tzivion et al., 1998; McPherson et al., 1999).

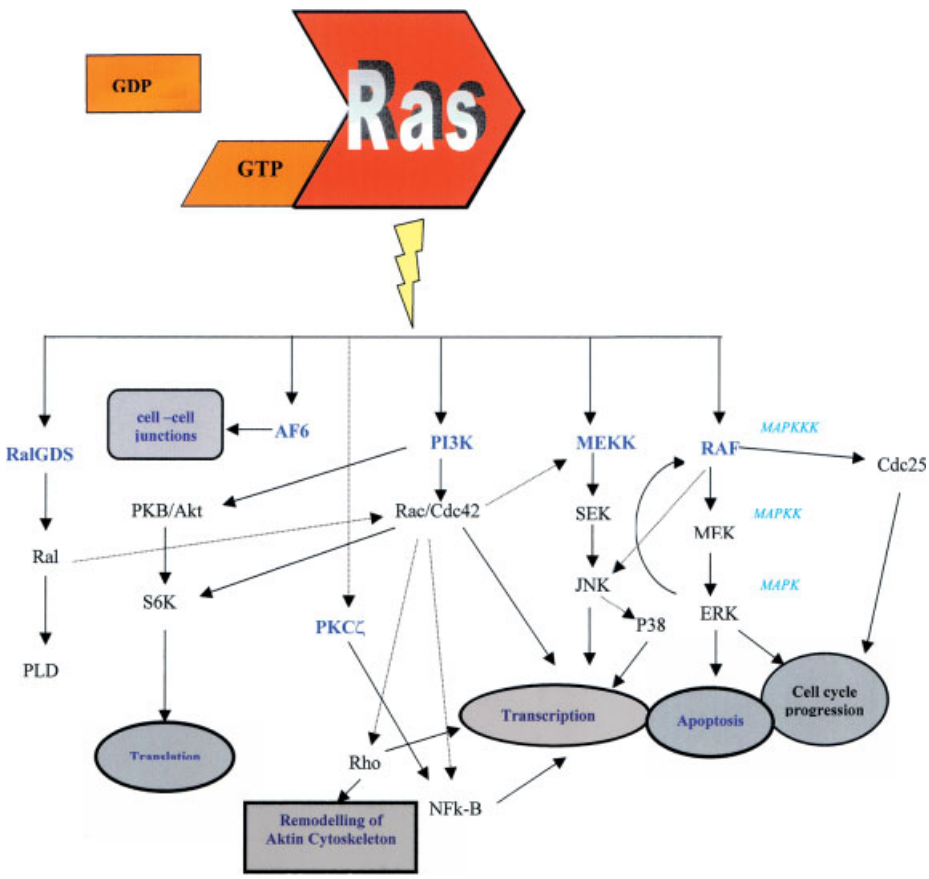

Fig. 2. Ras effectors and downstream pathways.

The RAF/Ras binding is transitory and once attached to plasma membrane, RAF activity becomes independent from Ras and is no longer influenced by its negative dominant mutations. The phosphorylated RAF activates a series of kinases in cascade that, amplifying low surface signals, modulate the activity of several cytoplasmatic and nuclear factors. Finally, the signals transmitted to the nucleus determine the activation of transcriptional factors, as the members of Ets family (Wasylyk et al., 1998). These transcriptional factors influence the expression of specific genes codifying for proteins involved in the control of cellular proliferation and/or differentation. For example, ERK1 and ERK2 kinases are involved in the induction of the c-fos expression through the ternary complex factor (TCF) phosphorylation, which bind the promoter of this gene. The product of c-fos, AP-1 transcription factor member, modulates the expression of many other genes. The same cascade of kinases stimulated by Ras/RAF complex induces cellular proliferation, and the expression of at least of two cyclin-dependent kinases inhibitors, $p21^{WAF1}$ and $p16^{INKa}$ (Lloyd, 1998). Ras induces apoptosis in some cellular types and in specific conditions through the same transduction pathway (Downward, 1998). In some cases, Ras stimulated apoptotic signals can be blocked by contemporary activation of Ras dependent transcriptional factor NF-kB (Mayo et al., 1997). Other biological responses mediated by Ras activation, include cellular movement, and cytoskeleton remodeling, among others.

Another Ras effector is the GEFs family (RalGDS, RGL, and Rlf/RGL2) that serve as activators of the Ral small monomeric GTPases (Wolthuis and Bos, 1999; Danen and Yamada, 2001). RAL-GTP activates the phospholipase D (PLD) that, by hydrolyzing phosphatidilcolines, generates satures and monoinsatures phosphatidates, which are putative activator molecules of Rho. RAL also seems to interact with a Cdc42 and RAC GAP (Hodgkin et al., 1998). Rho, RAC, and Cdc42 constitute another family of monomeric G proteins that play an important role in cytoskeleton remodeling and activate the kinases regulating the activity of various transcriptional factors. It has been shown recently that Rho represses expression of a cyclins inhibitor $p21^{WAF1}$ (Olson et al., 1998).

The third Ras effector is the phosphatidylinositol 3-kinase (PI3-K). Ras-GTP can bind and activate the catalytic subunit of this enzyme that generates PI(3,4,5)P3 by phosphorylation the PI(4,5)P2 in 3-position. The PI(3,4,5)P3 acts directly like a second messenger, binding several cytoskeleton kinase proteins and modulating the activity by conformational changes and/or their membrane translocation. PKB/AKT is an enzyme indirectly activated by PI3-K, which inactivates BAD, pro-apoptotic factor, by phophorylation (Downward, 1998).

Other potential Ras effectors are AF-6, protein kinase C-zeta (PKC-zeta), and Nore1. AF-6 was identified as a component of a critical fusion protein in certain leukemias (Taki et al., 1996; Watari et al., 1998). The function of AF-6 in epithelial cells is the formation of tight junctions between cell and its function is reduced in the presence of Ras (Yamamoto et al., 1997). Ras may use this effector to modulate intercellular binding and communication.

Another effector is PKC-zeta (Diaz-Meco et al., 1994; Liao et al., 1997) which display homology with Raf. Recent studies suggest that PKC zeta can activate Ras pathway independently of Ras (Anrather et al., 1999). Nore1 has been recently identified but its function remains unknown (Vavvas et al., 1998).

These recent observations begin to clarify the complex relationship between Ras activation, cellular proliferation, and apoptosis. In fact, Ras can induce simultaneously anti/pro-apoptotic pathways in function of quality and intensity activating signals, in function of cell type and metabolic conditions, and by activation of others Ras-independent or dependent pathways. A greater understanding of these processes would help identify the factors directly responsible for cell cycle deregulation in several tumors and would help the design of specific therapeutic strategies, for the control on the proliferation of neoplastic cells, or apoptosis.

RAS AND HUMAN CANCER

The important role of Ras in the regulation of cell growth and differentiation is verified by the fact that approximately 70% of neoplasias display, at different levels, mutations in this gene, especially in the 12 e13 codons that are the most examined sites in numerous studies. Other frequently mutated codons are 59 and 61. In vivo mutations in ras genes are not equally distributed between the ras isoforms. The K-ras gene is mutated in nonsmall cell lung cancer (33%), colorectal cancer (44%), and pancreas cancer (90%); N-ras is mutated in melanoma (13%), liver cancer (30%), and acute myelogenous leukemia (30%); H-ras gene is mutated in bladder cancer (10%) and kidney cancer (10%). Thyroid carcinomas have mutations in all three ras genes. In addition, numerous studies on hematological malignancies, pancreatic, colorectal, and nonsmall cell lung cancer have evaluated the potential role of Ras mutations as a negative prognostic factors but these results are conflicting (Field and Spandidos, 1990; Beaupre and Kurzock, 1999; Nelson et al., 1999; Pajkos et al., 2000; Russo et al., 2001).

The mutations in ras genes produce a oncoprotein which has been involved in tumor metastasis and angiogenesis. The tumor growth is dependent on the perpetual recruitment of host blood vessels (mainly via angiogenesis) to the tumor site. This process is thought to be triggered, at least in part, by the activation of oncogenes, inactivation/lost tumor suppressor genes. Potent oncogenes are able to deregulate expression of both angiogenesis stimulators and inhibitors in cancer cells. For example, genetic disruption of the mutant K-ras allele in human cancer is associated with increased production of vascular endothelial growth factor (VEGF) and downregulation of thrombospondin-1 (TSP-1). Upregulation of VEGF and angiogenesis can also be induced by constitutive activation of other oncogenic proteins (e.g., EGFR, Raf, MEK, PI3K) acting at various levels on the Ras signaling pathway (Rak et al., 2000).

RAS AND CELL CYCLE

Endogenous Ras is important for cell cycle progression and oncogenic Ras protein promotes growth

factor-independent cell cycle entry (Stacey and Kung, 1984; Mulcahy et al., 1985). Mitogen stimulation of quiescent cells (G0) causes two peaks of Ras activation, the first being necessary on entry into G1 phase and is associated with the Raf protein kinase cascade. Activation of the PI3K/Akt pathway corresponds with the second peak at mid-G1 phase. Activated Ras by mitogen stimuli is essential for upregulation of cyclin D1 and $p21^{CIP1}$ and for downregulation of $p27^{Kip1}$ (Taylor and Shalloway, 1996; Gille and Downward, 1999). Cyclin D1 Ras dependent upregulation has been attributed mainly to the Raf/MEK/ERK pathway activation (Lavoie et al., 1996; Kerkhoff and Rapp, 1998). The regulation of cyclin D1 by Raf/MEK/ERK pathway is undisputed but recent studies also suggest the contribution of others Ras effectors pathways for cyclin D1 induction (Gille and Downward, 1999). In fact, cyclin D1 expression is dependent on PI3K activity. Finally the PI3K/Akt pathway may also increase cyclin D1 protein expression by enhanced translation of cyclin D1 mRNA (Muise-Helmericks et al., 1998).

Ras or Raf cause cell cycle arrest by induction of $p21^{CIP1}$ (Sewing et al., 1997; Woods et al., 1997). Ras upregulation of $p21^{CIP1}$ is partially mediated by upregulation of transcription (Olson et al., 1998). In contrast to $p21^{Cip1}$, $p27^{Kip1}$ mRNA levels are constant during the cell cycle and are regulated by translational controls and by ubiquitin-mediated proteolysis (Pagano et al., 1995; Hengst and Reed, 1996). The Raf/MEK/ERK pathway is, perhaps, the best characterized effector pathway causing the downregulation of $p27^{Kip1}$ by Ras. Moreover, inhibition of PI3K blocks growth factor-induced downregulation of $p27^{Kip1}$, suggesting a role for this effector in Ras-mediated downregulation (Aktas et al., 1997).

Recent studies suggest a specific link between Ras signals and Rb regulation in cell cycle progression. In fact, inhibition of Ras function causes the activation of hypophosphorylated Rb contributing to G1 arrest (Pruitt et al., 2000). The role of Ras in promoting cell cycle progression is distinct in cells exiting from G0 and in cells in continuing proliferation (Hitomi and Stacey, 2001).

Finally, the relationship between Ras and the cell cycle is not simple because there are cell-type differences in how Ras could regulate cell machinery.

Ras proteins are intracellular key transducers of growth signals regulated by cell surface receptors. A high percentage of human tumors harboring oncogenic ras mutants suggests that this oncoprotein could be an appropriate target for drug design. The major Ras therapy approaches, at present, are the prevention of Ras membrane localization by using isoprenylation inhibitors (End, 1999; Rowinsky et al., 1999), the inhibition of Ras downstream effectors such as Raf kinase (Wood et al., 1999) and MEK pathway (Sebolt-Leopold et al., 1999), and the inhibition of Ras protein expression by antisense oligonucleotides, rybozimes or RNA (DeLong et al., 1997; Adjei et al., 2000).

CONCLUSIONS

Understanding the pathways regulating cell growth and differentiation plays a critical role in clarifying the mechanisms, leading to oncogenesis in human diseases. The function of oncogenic Ras and the knowledge that Ras dependent signaling pathways are crucial in oncogenesis are well established and are described in this review.

Every day the number of downstream effectors of Ras continue to be identified and described, but, unfortunately, their function in this complex signal network is still far from clear. At present, we see just the tip of the iceberg, but future research, using new modern and intriguing technologies, will help in defining the molecular and the physiological bases of the orchestrating signaling pathways in the cell. The ras gene expression, in time and space, is an important actor in this complex scenario of cell-cycle machinery, partially described in this review, and it can be said that Ras offers an interesting link between cancer and cell cycle.

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