Pergamon

European Journal of Cancer Vol. 31A, Nos. 7/8, pp. 1051–1054, 1995
Elsevier Science Ltd
Printed in Great Britain
0959-8049/95 $9.50+0.00

0959-8049(95)00168-9

p21ras: An Oncoprotein Functioning in Growth Factor-induced Signal Transduction

J.L. Bos

p21ras is a small GTPase that functions as a molecular switch in intracellular signal transduction pathways activated by a large variety of growth factors. In 50% of colorectal tumours, one of the genes for p21ras is mutated resulting in a constitutive active protein. Recently, progress has been made in the elucidation of the signalling pathways in which p21ras is involved. After a ligand binds to growth factor receptors, in particular receptor tyrosine kinases, a guanine nucleotide exchange factor is activated, which results in p21ras in the GTP-bound form. This GTP-bound form of p21ras interacts with the protein kinase raf1 and induces the activation of a kinase cascade, resulting in various cellular responses. This kinase cascade is part of an integrated network of both positive and negative signalling events.

Key words: small GTPases, oncogenes
Eur J Cancer, Vol. 31A, Nos 7/8, pp. 1051–1054, 1995

INTRODUCTION

p21ras IS AN important mediator of growth factor-induced differentiation and proliferation. Signalling pathways from growth factor receptors, via p21ras, to several cellular effectors have been identified. p21ras is the general name for three proteins encoded by the *HRAS*, *KRAS* and *NRAS* proto-oncogenes. These proteins differ structurally at their C-termini only, and thus far few, if any, functional differences between the proteins have been observed. p21ras is a small GTPase that cycles between an active GTP-bound form and an inactive GDP-bound form, and functions as a molecular switch in signal transduction. In a large number of tumours, one of the genes for p21ras is mutated resulting in a constitutive active protein, and in colorectal cancer, mutated *RAS* genes have been found. Mutated *RAS* genes can be detected both in carcinoma tissue and in the villous type of adenomas in approximately 50% of cases [1]. In several tumours with both carcinomatous and adenomatous tissue, the *RAS* mutation has been found in both tissue types, indicating that usually the mutation occurs prior to the conversion to malignant carcinoma. In the smaller adenomas, the frequency of *RAS* mutations is much lower (approximately 10% of cases) than in the larger villous adenomas [2]. This may imply that, in the majority of the cases, the mutation occurs later in development or, alternatively, that mutation of the *RAS* gene is (partly) responsible for the development of a small fraction of the polyps, but polyps with a mutated *RAS* gene have a higher probability to progress to malignancy.

The first clues to the function of p21ras were obtained when p21ras function was studied by micro-injection of neutralising antibodies. These studies have shown that, in a variety of cell types, p21ras is required for growth factor-induced DNA synthesis and gene expression [3], as well as for the induction of differentiation, i.e. nerve growth factor (NGF)-induced neurite outgrowth in PC12 cells. A second approach to studying the function of p21ras has been the use of dominant negative (interfering) mutants of p21ras [4]. One commonly used interfering mutant is $p21ras^{asn17}$. This mutant displays a reduced affinity for GTP, but normal affinity for GDP, and interferes in the GDP–GTP cycling of normal p21ras. Introduction of this mutant also inhibits ligand-induced proliferation and differentiation in various cell types. All these results point to a function of p21ras in growth factor receptor-mediated signal transduction.

RECEPTOR TYROSINE KINASES ACTIVATE p21ras

Convincing evidence that receptor stimulation can indeed activate p21ras has been obtained by experiments showing that epidermal growth factor (EGF) and insulin treatment result in a shift from p21rasGDP to p21rasGTP [5, 6]. The activation of p21ras is rapid and parallels the autophosphorylation of receptor. Activated EGF and insulin receptors are not unique in their ability to activate p21ras, and most, if not all, factors that activate receptor tyrosine kinases have been shown to activate p21ras. Ligands that activate (non-receptor) tyrosine kinases indirectly or certain members of the seven transmembrane receptor families also induce the activation of p21ras. However, the level and duration of p21ras activation depends on the cell type and the growth factor receptor that is activated.

MECHANISM OF p21ras ACTIVATION

Two rate-limiting steps can be distinguished in the cyclic regulation of the nucleotide content of p21ras. First, the dissociation of GDP followed by the binding of GTP, and secondly, the hydrolysis of p21ras-bound GTP. p21ras exhibits a slow intrinsic guanine nucleotide exchange and a slow intrinsic GTPase activity. However, guanine nucleotide exchange proteins regulate rapid GDP/GTP exchange, and GTPase activating proteins (GAPs) are present for the stimulation of GTP hydrolysis (Figure 1). p21ras can be activated by one of two mechanisms: an increase in guanine nucleotide exchange activity and/or an

Correspondence to J.L. Bos at the Laboratory for Physiological Chemistry, Universiteit Utrecht, Stratenum, P.O. Box 80042, 3508 TA Utrecht, The Netherlands.

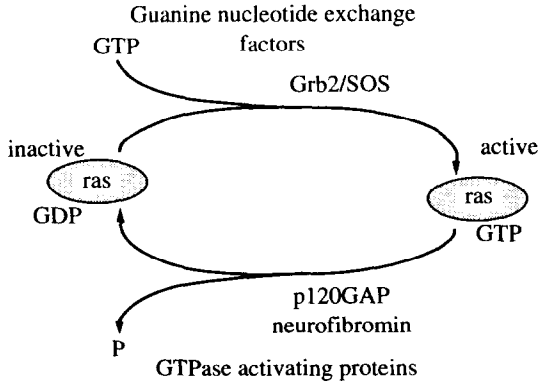

Figure 1. The p21ras cycle. p21ras cycles between an inactive GDP-bound and an active GTP-bound conformation. The cycle is regulated positively by guanine nucleotide exchange factors such as rasGRF and mSos and regulated negatively by GTPase activating proteins such as p120GAP and neurofibromin. Neurofibromin is the gene product of the neurofibromatosis type 1 gene.

inhibition of GAPs. For several reasons, initial attention focused on the possibility that a GAP was involved in the activation of p21ras. Indeed, following platelet derived growth factor (PDGF) stimulation, p120GAP rapidly associates with the activated PDGF receptor and is phosphorylated on tyrosine residues [7]. However, the function of p120GAP and its tyrosine phosphorylation in receptor tyrosine kinase signalling is still elusive.

Guanine nucleotide exchange on p21ras can be analysed by measuring the rate of labelled nucleotide binding to p21ras in permeabilised cells. Using this approach, it has been found that both insulin and EGF can induce a 2–3-fold increase in nucleotide binding to p21ras [8]. In the elucidation of the signalling pathway from growth factor receptor to p21ras, the genetic analysis of lower eukaryotes has been crucial, particularly the analysis of vulval development in *Caenorhabditis elegans* and eye development in *Drosophila melanogaster* (for references, see [9]). In both developmental processes, a receptor tyrosine kinase (Let23, Sevenless) receives an inductive signal and a p21ras homologue (Let60, Dras) serves as an intermediate to transduce signals to downstream targets. By genetic analysis, several genes have been identified that function in this pathway. One of these genes encodes Sem5, a protein containing an SH2 domain flanked by two SH3 domains. SH2 and SH3 domains were first identified as regions of homology between Src and Fps outside the kinase domain. SH2 domains are regions of approximately 100 amino acids that serve as a separate protein domain which can bind to phosphotyrosine-containing peptides. The specificity of binding is determined by the first few amino acids C-terminal of the phosphotyrosine. SH3 domains are smaller and bind to proline-rich sequences. Genetic evidence places Sem5 between Let23 and Let60. A protein homologous to Sem5 has been identified by virtue of either its ability to bind to the phosphorylated EGF receptor *in vitro* (Grb2) or its homology to SH2 domains.

In *Drosophila melanogaster*, a gene has been identified that shows identity with a yeast exchange factor for *RAS*. The encoded protein (Son of Sevenless or Sos) has been placed genetically between the Sevenless receptor and Dras. Surprisingly, both the *Drosophila melanogaster* Sos protein and the mammalian counterparts mSos1 and 2, contain a proline-rich sequence at the C-terminal end of the protein. This has led to the hypothesis that Grb2 functions as an adaptor protein, which can bind to phosphotyrosine residues of the receptor through its SH2 domain, and to the proline-rich segment of mSos through its SH3 domains. Indeed, it has been shown in *Drosophila melanogaster* that the Sem5 homologue Drk binds to both the Sevenless receptor and to Sos. This model has been tested for the EGF receptor [10]. It was found that a Grb2–mSos complex associates with the EGF receptor after EGF treatment, resulting in the translocation of the mSos protein to the particulate fraction of the cell. Interestingly, inhibition of the Grb2–EGF receptor interaction, by a phosphopeptide that mimics the Grb2 binding site on the EGF receptor, inhibits EGF-induced guanine nucleotide exchange in permeabilised cells [11]. This strongly suggests that binding of Grb2-mSos to the EGF receptor triggers the activation of p21ras. It should be noted that the association between Grb2 and mSos appears to be constitutive, and may not be, or only partially, induced by EGF treatment.

An alternative pathway for the EGF receptor to recruit p21ras is through the Shc protein. Shc is a protein containing an SH2 domain and a glycine/proline-rich sequence. It binds to the activated EGF receptor, and is phosphorylated on tyrosine residues upon EGF receptor activation [12]. In addition, upon EGF treatment, Grb2 associates with Shc. Therefore, it is possible that the observed interaction between the EGF receptor and mSos is (in part) mediated by Shc [13]. The possibility that Shc functions in p21ras signalling is supported by the observation that Shc overexpression induces p21*ras*-dependent neurite outgrowth in PC12 cells [14].

Adding to this complexity, is the observation that Syp, a phosphotyrosine phosphatase, can also bind to Grb-2-mSos [15]. All these interactions may recruit mSos to the plasma membrane, the location of p21ras, or induce a conformational change of mSos that results in the activation of guanine nucleotide exchange activity.

DOWNSTREAM EFFECTS OF GROWTH FACTOR-INDUCED ACTIVATION OF p21ras

p21ras has been implicated in the activation of a variety of cellular proteins. The first demonstration in mammalian cells that p21ras mediates insulin-induced cellular responses came from studies using the interfering mutant p21ras^{asn17}. In transient expression assays, this mutant inhibits insulin-induced activation of the *C-FOS*-promoter and the collagenase promoter [16]. Using a recombinant vaccinia virus with p21ras^{asn17} as a transgene, it was shown that expression of the interfering mutant inhibits insulin-induced activation of ERK2 kinase [17]. These results are part of a large series of reports on the signalling pathway from p21ras to downstream targets. Key enzymes in this pathway appear to be the serine/threonine kinases of the MAP2 kinase family [18]. These enzymes phosphorylate, at least *in vitro*, a variety of different cellular targets, and appear to play a crucial role in their activation. Among these targets are transcription factors, such as SRF (serum response factor) and *CMYC*, and other kinases, like MAPKAP kinase-2 and p90rsk [19]. In addition, ERK phosphorylates and activates phospholipase A2 [20] and may regulate through this pathway cytoskeletal rearrangements [21]. Indeed, this latter pathway, involving the small GTPases rac and rho seems to be most important for migratory events [22] including tumour invasiveness [23].

A picture of the signalling pathway between p21ras and ERKs is emerging (Figure 2). First, ERKs are phosphorylated on tyrosine and threonine residues, which results in the activation of the enzymes. A dual specificity kinase, MEK, is responsible for the phosphorylation, and thus for the activation. This kinase

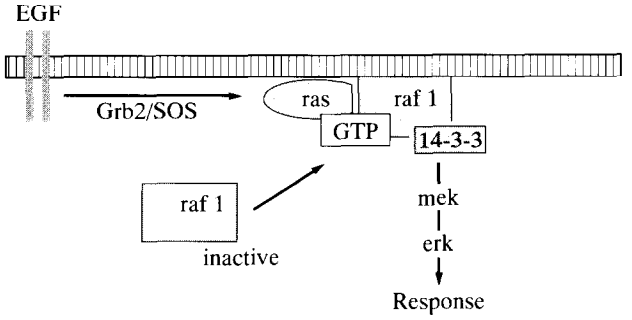

Figure 2. Activation of raf1 by activated p21ras. Receptor tyrosine kinases activate p21ras. Active p21ras, p21rasGTP, associates to the serine threonine kinase raf1, which is translocated to the plasma membrane. By some, as yet, unknown mechanism raf1 is activated. A protein of the 14-3-3 family may be involved in this process. Subsequently, MEK is phosphorylated and activated, followed by phosphorylation and activation of ERK.

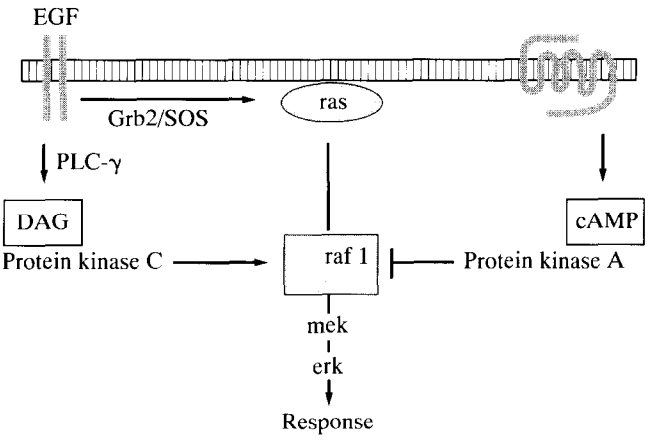

Figure 3. Agonists and antagonists of p21ras-mediated signalling. Receptor tyrosine kinases may activate raf1 via different pathways, one of which is p21ras, another protein kinase C (PKC). Activation of raf1 triggers the activation of MEK and ERKs. Certain serpentine receptors may activate raf1 through p21ras, but may inactivate raf1 through protein kinase A (PKA). For further explanation, see text.

is also activated by phosphorylation, in particular by members of the raf1 kinase family.

raf1 is encoded by the *CRAF* proto-oncogene. The 70 kDa raf1 protein is phosphorylated on several serine and, in some cases, also on tyrosine residues after growth factor stimulation. Analogous to oncogenic p21ras, *v-raf* is able to transform established cell lines. Inhibition of p21ras by neutralising antibodies or by interfering mutants of p21ras, does not abolish *v-raf* transformation, indicating that *v-raf* transforms cells independently of p21ras. An interaction between p21ras and raf1 was observed first by Moodie and associates [24], showing that raf1 could interact with p21rasGTP *in vitro*, but not or with reduced efficiency with p21rasGDP. Subsequently, it was shown that the p21ras and raf1 interaction results in the translocation of raf1 from the cytosol to the plasma membrane. Association of raf1 with p21ras-GTP, however, is not sufficient to activate raf1, and another signal may be required to establish full activation of raf1. Recently, one such protein has been identified, a chaperone-like molecule called 14-3-3 [25]. This protein is a member of a family of abundant proteins which bind to a variety of different proteins. The precise role of 14-3-3 in p21ras-mediated activation of raf1 is still unclear.

INTERFERENCE IN p21ras SIGNALLING

The role of protein kinase C in p21ras signalling has been a matter of debate. It has been postulated that protein kinase C is involved in growth factor-induced activation of p21ras as well as in growth factor-induced activation of ERKs. Although many different PKC isoenzymes are present, in NIH-3T3 cells there is no evidence that PKC is involved in insulin-induced activation of ERK2. Downregulation of PKC by prolonged pretreatment with the phorbol ester TPA or inhibition of PKC with H7 or sphingosine does not affect insulin-induced activation of p21ras or ERK2 [17]. Activation of PKC by TPA does activate ERKs. Apparently, PKC mediates a separate pathway to activate ERK2, which is operative in certain cell types and with certain ligands only (Figure 3). Indeed, EGF can activate ERK2 through different pathways. One of these pathways is mediated by p21ras, the other by PKC and a third by a calcium-dependent factor. The use of each of these pathways by EGF is cell type-dependent [26].

Growth factor-induced activation of p21ras signalling can be antagonised by signals that elevate the levels of cAMP, thereby activating PKA [27]. This leads to an inhibition of growth factor-induced activation of raf1 (Figure 3). The inhibition is cell type-specific, i.e. it occurs in NIH/3T3 and raf1 fibroblasts, but not in Swiss-3T3 fibroblasts and PC12 cells. Surprisingly, this inhibition of raf1 reflects the inhibition of cell growth by cAMP in these cells, suggesting that inhibition of raf1 is (part of) the molecular basis of cAMP-induced inhibition of cell growth.

The inhibition of raf1 also affects TPA-induced activation of ERK2, supporting the notion that PKC activates the ERK2 pathway by activating raf1. The inhibition of raf1 may be due to direct phosphorylation of ser 43 by PKA, which reduces the affinity of raf1 to p21rasGTP [28]. Alternatively, Rap1, which is a substrate for PKA and which can antagonise p21ras signalling, may be involved in this process.

FUTURE PROGRESS

From the above discussion, it is clear that p21ras mediates an important signalling pathway which triggers various events in the cell. Furthermore, this signalling pathway is subject to both positive (PKC) and negative (PKA) interference. Mutations in the p21ras gene result in the constitutive activation of this pathway. A number of the early steps that lead to early responses have been elucidated during the last few years. However, little is known about the steps that lead to later events, for instance, the intermediate steps before the induction of DNA synthesis, a process that starts 8 h after the induction of cell proliferation. Also, a connection between p21ras signalling and survival of cells from apoptosis has been implicated [29], but details are still lacking. Finally, the connection of p21ras with cytoskeletal rearrangements, motility and invasiveness is an intriguing field just being explored. Elucidation of these pathways is crucial to understand fundamental questions in cancer biology, and to design future therapeutic approaches.

1. Bos JL. *ras* oncogenes in human cancer: a review. *Cancer Res* 1989, **49**, 4682–4689.
2. Vogelstein B, *et al.* Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988, **319**, 525–532.
3. Stacey DW, Kung H-F. Transformation of NIH 3T3 cells by microinjection of Ha-ras p21 protein. *Nature* 1984, **310**, 508–511.
4. Feig LA, Cooper GM. Inhibition of NIH 3T3 cell proliferation by

a mutant ras protein with preferential affinity for GDP. *Mol Cell Biol* 1988, **8**, 3235–3243.
5. Gibbs JB, *et al.* Modulation of guanine nucleotides bound to ras in NIH3T3 cells by oncogenes, growth factors, and the GTPase activating protein (GAP). *J biol Chem* 1990, **265**, 20437–20442.
6. Burgering BMT, *et al.* Insulin stimulation of gene expression mediated by p21ras activation. *EMBO J* 1991, **10**, 1103–1109.
7. Molloy CJ, *et al.* PDGF induction of tyrosine phosphorylation of GTPase activating protein. *Nature* 1989, **342**, 711–714.
8. Medema RH, *et al.* Ras activation by insulin and epidermal growth factor through enhanced exchange of guanine nucleotides on p21*ras*. *Mol Cell Biol* 1993, **13**, 155–162.
9. Pronk GJ, Bos JL. The role of p21ras in receptor tyrosine kinase signalling. *Biochim Biophys Acta* 1994, **1198**, 131–147.
10. Egan SE, Weinberg RA. The pathway to signal achievement. *Nature* 1993, **365**, 781.
11. Buday L, Downward J. Epidermal growth factor regulates $p21^{ras}$ through the formation of a complex of receptor, Grb2 adaptor protein, and SOS nucleotide exchange factor. *Cell* 1993, **73**, 611–620.
12. Pelicci G, *et al.* A novel transforming protein (SHC) with an SH2 domain is implicated in mitogenic signal transduction. *Cell* 1992, **70**, 93–104.
13. Pronk GJ, *et al.* Involvement of Shc in insulin- and epidermal growth factor-induced activation of $p21^{ras}$. *Mol Cell Biol* 1994, **14**, 1575–1581.
14. Rozakis-Adcock M, *et al.* Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature* 1992, **360**, 689–692.
15. Li W, *et al.* A new function for a phosphotyrosine phosphatase: linking Grb2-Sos to a receptor tyrosine kinase. *Mol Cell Biol* 1994, **14**, 509–517.
16. Medema RH, Wubbolts R, Bos JL. Two dominant inhibitory mutants of p21*ras* interfere with insulin-induced gene expression. *Mol Cell Biol* 1991, **11**, 5963–5967.
17. de Vries-Smits AMM, *et al.* Involvement of p21ras in activation of extracellular signal-regulated kinase 2. *Nature* 1992, **357**, 602–604.
18. Cobb MH, Robbins DJ, Boulton TG. Extracellular signal-regulated kinases: ERKs in progress. *Cell Regulation* 1991, **2**, 965–978.
19. Davis RJ. The mitogen-activated protein kinase signal transduction pathway. *J biol Chem* 1993, **268**, 14553–14556.
20. Lin LL, *et al.* cPLA2 is phosphorylated and activated by MAP kinase. *Cell* 1993, **72**, 269–278.
21. Peppelenbosch MP, *et al.* Growth factor-induced cytoskeletal reorganization requires a p21ras/raf kinase dependent activation of phospholipase A_2. *Cell* 1995, in press.
22. Hall A. Small GTP-binding proteins and the regulation of actin cytoskeleton. *A Rev Cell Biol* 1994, **10**, 31–54.
23. Habets GGM, *et al.* Identification of an invasion-inducing gene, tiam-1 that encodes a protein with homology to GDP-GTP exchangers for rho-like proteins. *Cell* 1994, **77**, 537–549.
24. Moodie SA, *et al.* Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science* 1993, **260**, 1658–1661.
25. Morrison D. 14-3-3: modulators of signalling proteins? *Science* 1994, **266**, 56–57.
26. Burgering BMT, *et al.* cAMP antagonizes $p21^{ras}$-directed activation of extracellular signal-regulated kinase 2 and phosphorylation of mSOS nucleotide exchange factor. *EMBO J* 1993, **12**, 4211–4220.
27. Burgering BMT, Bos JL. Regulation of ras-mediated signalling: more than one way to skin a cat. *Trends Biochem Sci* 1995, **20**, 18–22.
28. Wu J, *et al.* Inhibition of the EGF-activated MAP kinase signalling pathway by adenosine 3′, 5′-monophosphate. *Science* 1993, **262**, 1065–1069.
29. Harrington EA, *et al.* c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. *EMBO J* 1994, **13**, 3286–3295.

Acknowledgement—The work in our laboratory is supported by the Dutch Cancer Society.