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Ras-Induced Transformation and Signaling Pathway

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Ras is a signal-transducing, guanine nucleotide-binding protein for various membrane receptors including tyrosine kinase receptors. Ras participates in the regulation of cell proliferation, differentiation, and morphology. Activated ras oncogenes have been identified in various forms of human cancer including epithelial carcinomas of the lung, colon, and pancreas. The cells of these cancers, as well as those that have been experimentally transformed by the activated ras gene, exhibit abnormal growth, morphological changes and alterations of cell adhesions. Although the main effector protein has been thought to be Raf serine/threonine kinase, research has revealed that the Ras-induced signaling pathway is mediated by multiple effector proteins and has the crosstalk with various factors containing other small GTPases. In this review, we summarize the involvement of each effector protein for Ras and the crosstalk with other small GTPases in Ras-induced transformation.

Key words: effector, GTPase, Ras, transformation.

Ras (H-Ras, K-Ras, N-Ras) is a signal-transducing small guanosine triphosphatase (GTPase) that plays central roles in the control of cell growth and differentiation (*1-3*). Ras has guanine nucleotide-binding activity and GTPase activities. Ras has GDP-bound inactive and GTP-bound active forms which are intercompatible by the GDP/GTP exchange and GTPase reactions. The GTPase reaction is regulated by GTPase-activating proteins (GAPs), such as p120 GAP, NF1, and Gap1. The GDP/GTP exchange reaction is regulated by guanine nucleotide exchange factors (GEFs). Ras is known to be the downstream molecule of receptor tyrosine kinases, such as EGF and PDGF receptors, and protein kinase C (*4-9*). The activation of these proteins results in the stimulation of GEFs and the conversion from the GDP-bound form to GTP-bound form of Ras. Figure 1 shows the well-known Ras-mediated signaling pathway. Activation of receptor tyrosine kinases recruits Sos guanine nucleotide exchange factor to the plasma membrane, mediating the association with an adaptor protein Grb2. The recruitment of Sos leads to the activation of Ras, followed by the activation of effector proteins. Recently two groups identified a Ras guanine nucleotide exchange factor with calcium- and diacylglycerol-binding motifs (namely Ras GRP or CalDAG-GEFII), suggesting the existence of a mechanism whereby the activation of Ras is regulated directly by the second messengers, calcium and diacylglycerol (*10, 11*).

Mutations in ras genes have all been found in human tumors, and the frequency of ras mutations is the highest among any genes in human cancers (*12, 13*). Ras-transformed fibroblasts show typical anchorage-independent growth and morphological change. These phenotypes are thought to be caused by Ras-induced gene expression and rearrangement of the cytoskeleton and cell adhesions. Although the Raf family members are the only effector proteins for which genetic evidence is available regarding activity as a candidate Ras effector, Ras-induced transformation can not be explained by the effect of the Raf family alone. Recently various candidates for Ras effector proteins have been reported (Fig. 2). We will summarize the putative roles of these effector proteins and other Ras-like small GTPases in Ras-induced transformation.

Ras effectors and Ras-induced transformation

Raf. Raf serine/threonine kinases (Raf-1, A-Raf, and B-Raf) are the best-characterized effector for Ras and the only one for which genetic evidence has been obtained. Growth factor-mediated or oncogenic activation of Ras recruits Raf to the plasma membrane, where it is activated by a mechanism which is not fully understood. Several observations revealed that the activation of Raf plays an important role in Ras-induced transformation (*14, 15*). The activated Raf phosphorylates and activates MEK (MAPK/ERK kinase). Subsequently, the activated MEK phosphorylates and activates MAPK (mitogen-activated protein kinase). The activated MAPK is translocated to the nucleus and activates several transcriptional factors such as TCF and Jun. Although Raf plays critical roles by deregulating the mitogenic signaling in Ras-induced transformation, some evidence indicate that Raf alone is not sufficient to cause the cytoskeletal and morphological changes in Ras-induced transformation (*16-18*). Raf-CAAX (Raf-1 kinase which is targeted to plasma membrane) fails to induce cytoskeletal rearrangement in some fibroblasts and endothelial cells under the conditions where activated Ras can induce membrane ruffling. Moreover, the involvement of other effectors in Ras-induced transformation was indicated by the fact that Raf-binding defective mutants of Ras, which can interact with other effectors, possess some but

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not full transforming activities.

Phosphatidylinositol 3-kinase. Phosphatidylinositol 3-kinase (PI 3-kinase) interacts with the activated Ras and is itself activated (*19-21*). PI 3-kinase is involved in the regulation of the actin cytoskeleton by growth factors such as PDGF and insulin (*22-24*). The experiments using various effector mutants of Ras revealed that the activation of PI 3-kinase is necessary for actin cytoskeletal rearrangement in the Ras-induced transformation and that it is mediated by the activation of Rac (*18*). Since the activated form of PI 3-kinase alone can not cause cellular transformation of fibroblasts, cooperative workers such as Raf family proteins appear to be necessary for the PI 3-kinase-mediated pathway in Ras-induced transformation.

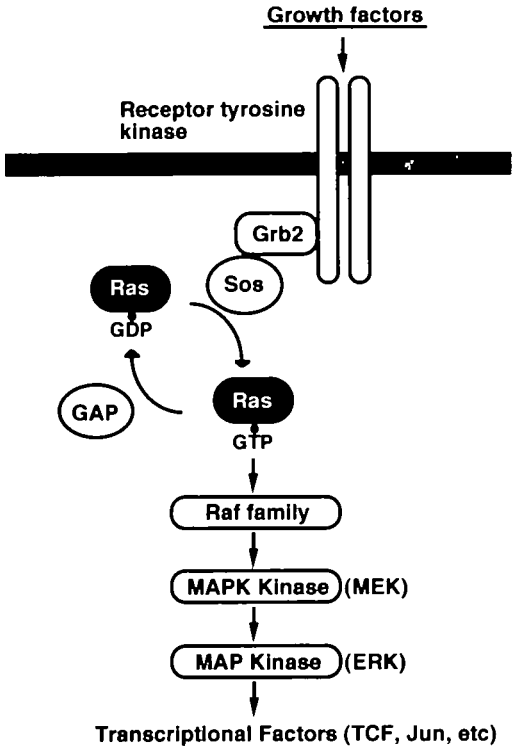

Fig. 1. **Ras signaling pathway.** Growth factor-mediated activation of receptor tyrosine kinases recruits Sos guanine nucleotide exchange factor to the plasma membrane through the association with an adaptor protein Grb2. The recruitment of Sos leads to the activation of Ras. The activated Ras recruits Raf to the plasma membrane and Raf is activated by a mechanism which is not fully understood. The activated Raf phosphorylates MEK (MAPK/ERK kinase) and activates it, following which the activated MEK phosphorylates MAP kinase (mitogen-activated protein kinase) and activates it (ERK). The activated MAPK is translocated to the nucleus and activates several transcriptional factors such as TCF and Jun.

Ral GEFs. The yeast two hybrid system revealed that Ral guanosine exchange factors (Ral GEFs) (RalGDS, RGL, RLF) bind to the activated Ras. The Ras-associating domain of RalGDS has low sequence similarity with that of Raf kinase, but it has been found that their overall three-dimensional structures are very similar to each other (*25*). Many proteins, including AF-6, RIN1 and PLC210 (see below), have been found to contain the Ras-associating domain which shows high sequence homology with that of RalGDS (Fig. 3). RalGDS stimulates the GDP/GTP exchange of Ral in a Ras-dependent manner in COS cells (*26, 27*). RalGDS and Raf synergistically stimulate cellular proliferation and gene expression (*28, 29*). These results suggest that the Ral GEF-Ral pathway contributes to the Ras-induced transformation.

AF-6. We previously identified the ALL-1 fusion partner from chromosome 6 (AF-6) as a novel Ras effector (*30*). AF-6 was identified as the fusion partner of acute lymphoblastic leukemia-1 (ALL-1) protein (*31*). The ALL-1/AF-6 chimeric protein is a critical product of the t(6;11) abnormality associated with some human leukemias. AF-6 binds to the activated Ras via its amino-terminal region *in vitro* (*30*) and also *in vivo* (*32*). AF-6 has the PDZ domain, which is thought to localize AF-6 at specialized sites of the plasma membrane such as cell-cell contact sites. AF-6 accumulates at various sites of cell-cell contact, such as cell-cell adhesion (*33, 34*), and synaptic junctions (*35*), suggesting that AF-6 plays a role in the regulation of cell-cell adhesion. Consistently, it has recently been shown that the absence of AF-6 disrupts epithelial cell-cell adhesion and cell polarity during mouse development (*36*). AF-6 interacts with ZO-1 which is one of the cell-cell adhesion molecules. ZO-1 interacts with the Ras-associating domain of AF-6, and this interaction is inhibited by the activated Ras (Fig. 4). The

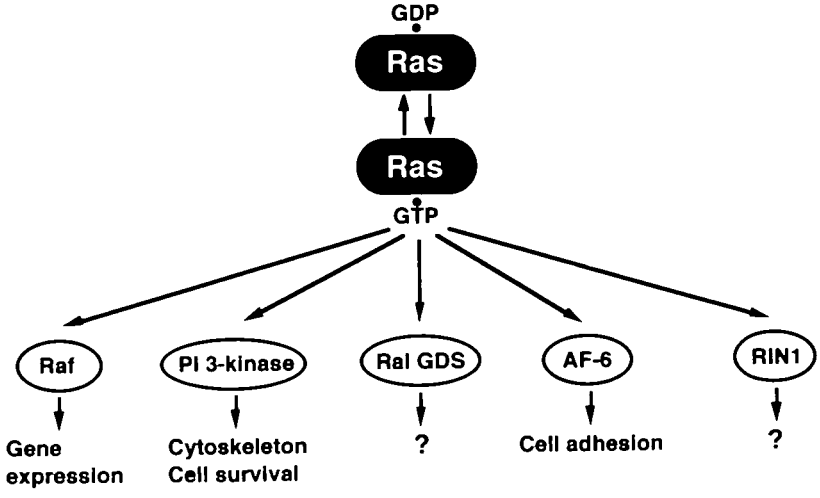

Fig. 2. **Ras effectors.** Ras contributes to various cellular processes and multiple effector proteins of Ras are involved in Ras-mediated signaling. They include Raf, PI 3-kinase, RalGDS, AF-6, and RIN1.

overexpression of activated Ras in Rat1 cells results in the perturbation of cell-cell contacts, followed by a decrease in the accumulation of AF-6 and ZO-1 at the cell surface (*34*). It remains to be clarified whether AF-6/ZO-1 interaction is really involved in the regulation of the cell-cell adhesion and the Ras-induced transformation.

Recently we identified Fam as an AF-6-interacting protein (*37*). Fam is homologous to a deubiquitinating enzyme in *Drosophila*, the product of the *fat facets* (*faf*) gene (*38*). Although the genetic interaction of *ras* with *fat facets* in *Drosophila* has been reported (*39, 40*), it remains to be clarified whether Fam is involved in the Ras-induced transformation and Ras signaling pathway in mammals.

PLC210. Recently a yeast two-hybrid screening identified PLC210, a *C. elegans* phospholipase C as a Let-60 Ras-binding protein (*41*). PLC210 contains a catalytic region highly homologous to that of the PI-PLC family proteins. This catalytic region contains the conserved amino acid residue for the catalytic activity and for Ca^{2+}-dependent interaction with phosphoinositides. PLC210 contains two Ras-associating domains conserved structurally among RalGDS and AF-6. PLC210 binds to Ras in a GTP-dependent manner via the region containing these domains, but it is unknown whether this interaction affects the PI-PLC activity of PLC210. In addition, PLC210 contains the amino-terminal CDC25-like domain. This domain is homologous to the catalytic domain of GDP/GTP exchange factors for Ras, especially to those of mammalian Sos2 and *Drosophila* Sos, suggesting that PLC210 is a bifunctional protein, which possesses two distinct catalytic activities. It is unclear whether Ras regulates the activity of PLC210 and whether a mammalian counterpart of *C. elegans* PLC210 exists. However, the observation that the phosphoinositide turnover rate was elevated in Ras-transformed cells implied the presence of unknown enzymes like PLC210 in mammals.

RIN1. RIN1 (Ras interaction/interference) was first identified as a Ras-binding protein that suppresses the activated *RAS2* allele in *S. cerevisiae* (*42, 43*). RIN1 binds to the activated Ras through its carboxyl-terminal domain (Fig. 3) and this Ras-binding domain also binds to 14-3-3 proteins as Raf-1 does. In addition, RIN1 contains two other functional domains, SH2 and proline-rich domain. The SH2 domain of RIN1 possesses the ability to interact with the phosphotyrosine-containing proteins, but the physiological partners for this domain are unknown. The proline-rich domain in RIN1 is similar to the consensus SH3 binding regions. Indeed, the amino-terminal region containing this proline-rich domain binds to the SH3 domain of c-ABL tyrosine kinase and is phosphorylated by c-ABL, but this interaction does not affect the c-ABL catalytic activity (*44*). These results indicate that RIN1 participates in multiple signaling pathways, but it remains to be clarified whether RIN1 is involved in the Ras-induced transformation.

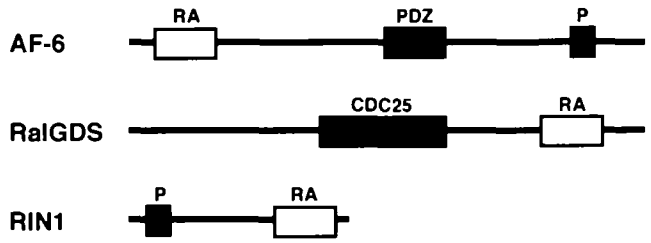

Fig. 3. **The proteins containing the Ras-associating domain.** The family of proteins containing a Ras-associating (RA) domain is increasing. Among them, AF-6, RalGDS, and RIN1 are shown to interact with the activated Ras. Abbreviation used in this figure: P, proline-rich domain.

Involvement of other small GTPases in Ras-induced transformation

Rap1. Rap1 was identified as an antagonist of Ras-induced transformation (*45*). Since Rap1 contains an effector region almost identical to that of Ras, it can interact with similar effectors such as Raf-1 and Ral GEF. These interactions may be utilized for trapping Ras effectors to antagonize Ras signaling. On the other hand, it has been reported that Rap1 has positive effects on mitogenesis and oncogenic transformation (*46*). Therefore, it should be further examined whether the function of Rap1 is positive or negative against Ras-induced transformation.

Ral. Ral consists of RalA and RalB. Since insulin and EGF-induced activation of Ral is inhibited by dominant negative Ras, the Ral pathway is a downstream signaling pathway of Ras (*47*). As described above, Ral GEF is one of the Ras effectors and Ral GEF-Ral pathway may contribute to the Ras-induced transformation. Surprisingly, although

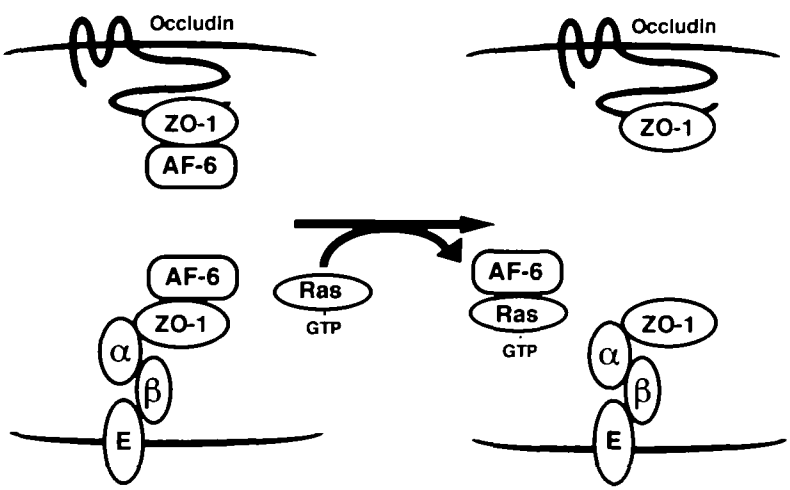

Fig. 4. **AF-6/ZO-1 interaction and Ras-induced transformation.** AF-6 is a peripheral protein at sites of cell-cell contact such as cell-cell adhesion. AF-6 interacts with ZO-1, a peripheral component of cell-cell adhesions. The activated Ras inhibits the interaction of AF-6 and ZO-1. The activated Ras may regulate the state of AF-6/ZO-1 complex in Ras-induced transformation. Abbreviations used in this figure: E, E-cadherin; α, α-catenin; β, β-catenin.

a dominant negative form of Ral blocks a Ras-dependent transformation in NIH3T3 cells, Ral^{V23} alone, a constitutively activated form of Ral, can not efficiently induce the oncogenic transformation as compared with the activated Ras and Ral GEF (*26, 48*). This observation suggests that the transformation induced by Ral GEF may require other factors in addition to Ral. Two effectors for Ral are known, RalBP1 and phospholipase D (PLD). RalBP1 interacts with Ral in a GTP-dependent manner and contains a RhoGAP homology domain which exhibits the GAP activity for Rac1 and Cdc42 but not for RhoA (*49-51*). Although Rac and Cdc42 contribute to the Ras-induced transformation as described below, it is unclear whether the association of Ral with RalBP1 regulates the activity of these GTPase. PLD is activated in v-Src transformation. Dominant negative RalA mutants inhibited both v-Src- and v-Ras-induced PLD activity suggesting the involvement of RalA in Ras/Src-induced PLD activation (*52*).

Rho family. A critical role for the Rho family GTPases in Ras-induced transformation is supported by a number of experimental observations (*53-59*). The Rho family regulates multiple signaling pathways that affect cell shape and motility, transcription, and cell-cycle progression. In the control of the actin-based cytoskeleton of fibroblasts, each member of the Rho family is implicated in the formation of a distinct structure: Cdc42 induces filopodia (*24, 60*), Rac regulates the formation of lamellipodia and membrane ruffling (*61*), and Rho is involved in the assembly of stress fibers and focal adhesions (*62*). Recently we found that the dominant active form of Rho (Rho^{V14}) reverts not only the formation of stress fibers and focal adhesions but also cell-cell adhesions and that constitutively activated Rho-kinase, a downstream effector of Rho, restores the assembly of stress fibers and focal adhesions in Ras-transformed Rat1 fibroblast, suggesting that the Rho-Rho-kinase pathway is inactivated in the cells expressing Ras^{V12}, and this may contribute to oncogenic Ras-induced transformation (*63*).

Perspectives

To understand the mechanism by which Ras induces transformation of certain types of cells, enormous efforts have been made over the last decade to identify downstream effectors of Ras. As a result, a number of the effectors have been isolated. Intensive analyses of their functions have provided some insights regarding the modes of action of Ras at the molecular level. However, the mechanism underlying Ras induced abnormal growth, morphological changes and alterations of cell adhesions remains to be clarified. Further studies will lead to a better understanding of how Ras induces transformation, including rearrangements of cytoskeleton and cell adhesion.

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