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Differential Functions of Ras for Malignant Phenotypic Conversion

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Among the effector molecules connected with the group of cell surface receptors, Ras proteins have essential roles in transducing extracellular signals to diverse intracellular events, by controlling the activities of multiple signaling pathways. For over 20 years since the discovery of Ras proteins, an enormous amount of knowledge has been accumulated as to how the proteins function in overlapping or distinct fashions. The signaling networks they regulate are very complex due to their multiple functions and cross-talks. Much attention has been paid to the pathological role of Ras in tumorigenesis. In particular, human tumors very frequently express Ras proteins constitutively activated by point mutations. Up to date, three members of the Ras family have been identified, namely H-Ras, K-Ras (A and B), and N-Ras. Although these Ras isoforms function in similar ways, many evidences also support the distinct molecular function of each Ras protein. This review summarizes differential functions of Ras and highlights the current view of the distinct signaling network regulated by each Ras for its contribution to the malignant phenotypic conversion of breast epithelial cells. Four issues are addressed in this review: (1) Ras proteins, (2) membrane localization of Ras, (3) effector molecules downstream of Ras, (4) Ras signaling in invasion. In spite of the accumulation of information on the differential functions of Ras, much more remains to be elucidated to understand the Ras-mediated molecular events of malignant phenotypic conversion of cells in a greater detail.

Key words: Ras, Tumorigenesis, Signaling, Invasion, Migration

INTRODUCTION

Normal cell proliferation in multicellular organism is tightly controlled to ensure that it occurs only when it is required. Break-down of normal growth regulation often leads to cancer. The Ras proteins are some of the first proteins identified that can regulate cell growth. Aberrant Ras function has been well acknowledged to be strongly associated with human cancer development. Biochemical and molecular approaches have been used to inhibit Ras-mediated oncogenic activities. A major drawback of these approaches is *in vivo* toxicity. Since Ras proteins are central molecules responsible for cell survival, proliferation and other cellular processes, inhibition of general Ras activities can be detrimental not only to cancer cells but also to normal cells. Therefore, it is critical to identify Ras downstream signaling molecules that are required for malignant cancer cell behavior but less critical for normal cell functions. The functions and signaling pathways of Ras proteins and therapeutic approaches to target Ras pathways are now understood in great detail as summarized in several excellent reviews (Magee and Marshall, 1999; Shields *et al.*, 2000; Downward, 2003; Hancock, 2003). The present review is aimed to highlight the current understanding of the differential functions of Ras proteins in the malignant phenotypic conversion of epithelial cells.

The following five themes are addressed in this review: **(1) Ras proteins.** It is generally assumed that there are three human *ras* genes that code for Ras proteins and there have been indications for different molecular functions of these proteins. The activation of different Ras isoforms can have distinct biochemical consequences for cells, rationalizing the mutation of specific Ras isoforms in different human tumors. **(2) Membrane localization of Ras proteins.** Since a considerable body of evidence has suggested that functional differences among the Ras isoforms could be due to variations in plasma membrane microlocalization, it is important to identify a link between interaction of Ras with the plasma membrane and the functional differences in Ras signaling. **(3) Ras downstream effector molecules.** Ras proteins exert biological

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activities by stimulating a multitude of downstream signaling cascades. This review summarizes the role of signaling molecules in Ras-induced cellular responses and the differential regulation of signaling pathways by Ras isoforms. **(4) Ras signaling in invasion.** It is well appreciated that the activated Ras proteins contribute significantly to several aspects of the malignant phenotypes including invasiveness, migration and angiogenesis. This review covers current understanding of tumor invasion with a major focus on the contribution of distinct signaling network exerted by different Ras isoforms to invasion and migration of breast epithelial cells.

(1) Ras proteins

The Ras proteins are guanine nucleotide-binding proteins that play an essential role in transducing extracellular signals to diverse cellular responses, including cell proliferation and differentiation (Boguski and McCormick, 1993; Campbell *et al.*, 1998). One of the most frequent defects in human cancer is the uncontrolled activation of the Ras-signaling pathways (Barbacid, 1987). Human tumors frequently express Ras proteins that have been activated by point mutations – about 20% of all tumors have undergone an activating mutation in one of the *ras* genes (Barbacid, 1987; Bos, 1989).

The Ras family consists of three identified members: Harvey-Ras (H-Ras), Kirsten-Ras (K-RasA and K-RasB), and N-Ras, proteins of 188-189 amino acids with a molecular weight of 21 kDa. Activating point mutation has been most frequently found in K-Ras (about 85% of total), then N-Ras (about 15%), then H-Ras (less than 1%) (Downward, 2003). While the N-terminal 85 amino acids are identical and the middle 80 amino acids contain 85-90% homology between Ras proteins, the C-terminal sequence, so-called the hypervariable region (HVR), is highly divergent (Barbacid, 1987; Boguski and McCormick, 1993). Homology of HVR among the four Ras proteins was shown to be 4-15% (Shield *et al.*, 2000; Prior and Hancock, 2001). The sequence divergence between the Ras proteins is shown in Fig. 1.

An important question on Ras biology is whether the ubiquitously expressed, almost identical Ras isoforms have distinct functions. There are differences in the signal transduction pathways induced by Ras proteins, supporting unique functions of Ras family members (Carbone *et al.*, 1991; Umanoff *et al.*, 1995; Johnson *et al.*, 1997; Koera *et al.*, 1997; Yan *et al.*, 1998). Although these Ras proteins share common signaling pathways leading to similar cellular responses, cell-type specific differences in the transforming potential between Ras proteins were also reported. One member of the *ras* family appears to be the preferred "target gene" in particular tumors. The single point mutation at amino acid residue 12 (glycine to aspartate) of H-Ras is more often found in mammary carcinoma, whereas the same mutation of N-Ras is detected in teratocarcinoma and leukemia (Franks and Teich, 1997). While H-Ras is more transforming than *N*- or K-Ras in murine fibroblasts, N-Ras is more transforming in human haemopoietic cells (Maher *et al.*, 1995). Differences in the transforming activitites of Ras proteins appear to be due to unique sequences between amino acids 84 and 143 in Ras proteins (Maher *et al.*, 1995). H-Ras, but not N-Ras, induces invasive and migrative phenotypes in human breast epithelial cells while both H-

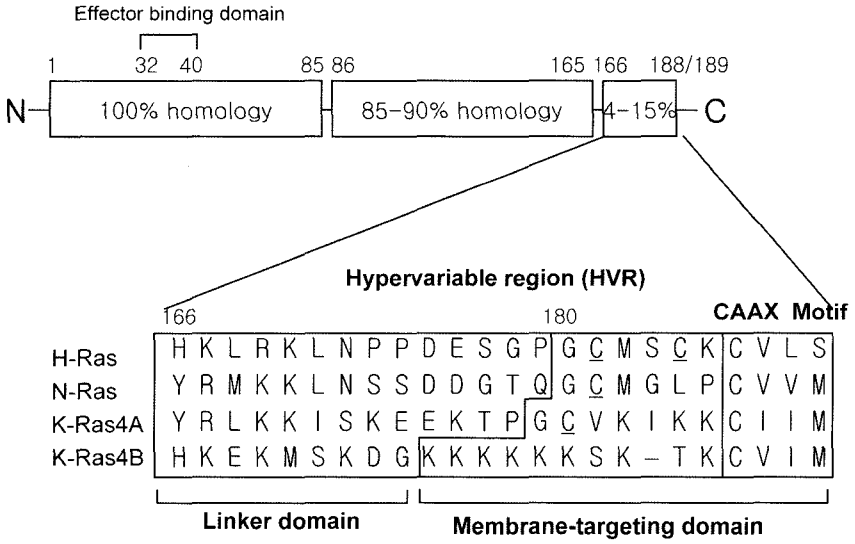

Fig. 1. Sequence divergence between Ras isoforms. The Ras proteins show complete homology in their amino-terminal 85 residues. The greatest sequence divergence is seen in the carboxy-terminal residues of 165-188/189, a region called hypervariable region (HVR). Cysteine palmitoylation sites are underlined.

Ras and N-Ras induce transformed phenotypes such as focus-forming morphological change and anchorage-independent growth (Moon *et al.*, 2000; Kim *et al.*, 2003).

(2) Membrane localization of Ras proteins

Since dynamic microdomain localization has implications for understanding how signaling complexes are assembled and disassembled in response to particular stimuli, it is important to identify a link between interaction of Ras with the plasma membrane (Magee and Marshall, 1999) and the functional differences in Ras signaling. The differences appear to result, at least in part, from differences in the mechanisms of membrane attachment of the three Ras isoforms (Yan *et al.*, 1998; Booden *et al.*, 2000).

The biological activity of Ras requires a proper localization to the inner surface of the plasma membrane (Willumsen *et al.*, 1984). Several studies, however, demonstrate that Ras is also activated on and transmits signals from the endoplasmic reticulum and Golgi apparatus, suggesting that the plasma membrane may not be the exclusive platform from which Ras regulates signaling (Chiu *et al.*, 2002; Bivona *et al.*, 2003). Accumulating evidence has revealed differences in the way that Ras proteins are routed to the plasma membrane (reviewed in Hancock, 2003). Such differences seem reasonable since the HVR contains the signals responsible for the correct localization of Ras (Fig. 1). These signals include the C-terminal CAAX box (in which A = aliphatic amino acid and X = serine or methionine) (Casey *et al.*, 1989; Gutierrez *et al.*, 1989) and palmitoylation of two cysteine residues (Cys^{181} and Cys^{184}) in H-Ras, one cysteine (Cys^{181}) in N-Ras, and a polybasic sequence of multiple lysines ($Lys^{175-180}$) in K-Ras (Hancock *et al.*, 1990; Kato *et al.*, 1992). These moieties and possibly the entire HVR sequence confer membrane-anchoring capacity on Ras and are also involved in the membrane trafficking of Ras proteins (Choy *et al.*, 1999).

Given that correct post-translational modification of Ras proteins is required for their biological activities, several strategies have been developed to inhibit the membrane anchoring of Ras proteins. The enzymes involved in this processing have become potential targets for therapeutic intervention (Seabra *et al.*, 1998; Cox and Der, 1997; Hancock *et al.*, 1989; Hancock *et al.*, 1990), the most common being the design of compounds that mimic the CAAX motif and compete for binding to farnesyltransferase (Cox and Der, 1997; Oliff, 1999; Downward, 2003). These farnesyltransferase inhibitors (FTI) as tumor therapeutic drugs, however, turned out to be selective for H-Ras since K-Ras and, to a lesser extent, N-Ras can also be modified by geranylgeranyltransferase.

The interactions of Ras proteins with plasma membrane can differ from one isoform to another due to their different membrane anchoring moieties. Several recent studies (Niv *et al.*, 2002; Prior *et al.*, 2003; Roy *et al.*, 1999; Parton and Hancock, 2004) revealed that differently anchored Ras proteins display different interactions with lipid rafts (Simons and Toomre, 2000), which are cholesterol/sphingolipid-enriched microdomains that dynamically organize specific membrane proteins. H-Ras (wild type) but not K-Ras is significantly concentrated in cholesterol-dependent rafts (Niv *et al.*, 2002). Differential activation of Raf-1 and phosphatidylinositol 3-kinase (PI3K) by K-Ras and H-Ras (Yan *et al.*, 1998) may be explained by the differences in the microdomain localization of these Ras proteins (Yan *et al.*, 1998; Prior *et al.*, 2001; Matallanas *et al.*, 2003). Mutation of H-Ras C terminus changed effector pathway utilization (Booden *et al.*, 2000), suggesting a role of the lipidated C-terminal in the biological functions of Ras proteins. These studies demonstrate that the localization of Ras proteins to different microdomains of the plasma membrane may be critical for signaling specificity. It has been demonstrated that H-Ras and N-Ras differentially regulate Rac1 activity which plays a key role in invasion and migration while they do not vary in their abilities to activate Raf-1 and PI3K in MCF10A cells (Shin *et al.*, 2005). Detailed comparison of microlocalization of H-Ras and N-Ras needs to be performed to investigate whether differential microlocalization in plasma membrane accounts for the distinct regulation of signaling pathway and invasive phenotype by the highly homologous H-Ras and N-Ras proteins.

(3) Ras downstream effector molecules

Ras proteins exert their biological effects by activating several downstream effector molecules including Raf, PI3K and Ral (Marshall, 1996; Campbell *et al.*, 1998). The first mammalian effector of Ras is the serine/threonine kinase Raf (Leevers *et al.*, 1994). GTP-Ras binds to Raf and this interaction causes Raf relocation to the plasma membrane which seems to be critical for its activation (Marais *et al.*, 1995). Ras-stimulated Raf activates the downstream kinase MAPK/ERK (MEK), which in turn phosphorylates extracellular signal-regulated kinases (ERKs) (Kyriakis *et al.*, 1992; Alessi *et al.*, 1994). ERKs can be transported into the nucleus following activation, stimulating nuclear transcription factors such as Ets family Elk1, Fos and Jun. The Raf-MEK-ERK pathway can mediate Ras-induced cell survival and proliferation by promoting cell-cycle progression (Yordy *et al.*, 2000; Pruitt and Der, 2001; Vaudry *et al.*, 2002).

In addition to the Raf-MEK-ERK pathway, the biological effects of Ras proteins are exerted through the activation of PI3K (Downward, 1998). Ras can directly interact with the catalytic subunit of PI3K and activate the molecule (Rodriguez-Viciana *et al.*, 1994; Pacold *et al.*, 2000). PI3K

controls the activity of a large number of downstream molecules by phosphorylating phosphatidylinositol 4, 5-bisphosphate to produce phosphatidylinositol 3, 4, 5-triphosphate which is a second messenger that binds to a number of proteins. Much attention has focused on the activation of Akt (also called protein kinase B) which has a strong anti-apoptotic function (Romashkova and Makarov, 1999) and seems to be an important mediator of Ras-generated survival signal (Downward, 1998; Datta *et al.*, 1999; Khwaja *et al.*, 1997; Khwaja, 1999). Ras also activates three GDP-GTP exchange factors (RalGDS, RGL and RGL2/Rlf) to stimulate Ral (Feig *et al.*, 1996; Wolthuis and Bos, 1999).

The small GTP-binding protein Rac has been shown to be critical for the mitogenic and oncogenic effects of Ras by promoting actin cytoskeletal reorganization leading to membrane ruffling, lamellipodia formation, cell migration and invasion (Hancock *et al.*, 1990; Ridley *et al.*, 1992; Qiu *et al.*, 1995; Joneson *et al.*, 1996; Etienne-Manneville and Hall, 2002). Rac can be responsible for Ras-induced changes in the actin cytoskeleton associated with developing invasive carcinoma of mammary epithelial cells by modulating motility and invasion (Nobes *et al.*, 1995). Rac activation, which can occur through PI3K-dependent and PI3K-independent pathways, is important in Ras-induced transformation and invasion (Lambert *et al.*, 2002; Malliri *et al.*, 2002).

The signaling order between Rac and PI3K has been controversial in different cell systems. PI3K acts upstream of Rac1 in pathways for membrane ruffling, chemotaxis (Reif *et al.*, 1996; Welch *et al.*, 2003) and inducing malignant phenotype of signet-ring cell carcinoma (Xu *et al.*, 2003). Rac1 has been shown to act upstream of PI3K to promote cellular motility and invasiveness by disrupting the normal polarization of mammary epithelial cells (Keely *et al.*, 1997; Sachdev *et al.*, 2002). In H-Ras-activated MCF10A cell system, PI3K pathway was dependent on Rac activity while Rac activity was not affected by PI3K inhibition, suggesting that Rac1 may lie upstream of PI3K (Shin *et al.*, 2005).

The reason why the Ras proteins need many downstream effector molecules has been speculated (Shields *et al.*, 2000). Ras exerts a diverse spectrum of cellular responses depending on different cell systems. Utilization of distinct sets of multiple signaling pathways is required for the complex nature of the transformed phenotype exerted by oncogenic Ras, such as uncontrolled proliferation, loss of anchorage-dependent growth, invasion, metastasis and angiogenesis.

Despite the fact that the amino acid sequence corresponding to the effector binding loop, which spans residues 32-40, is identical among Ras proteins (Fig. 1), recent studies have demonstrated that the three Ras isoforms can differentially activate the effector molecules. There are differences in the signal transduction pathways induced by Ras proteins, suggesting unique functions of different Ras family members at the molecular level (Carbone *et al.*, 1991; Voice *et al.*, 1999). K-Ras activates Rac more effectively than H-Ras (Walsh and Bar-Sagi, 2001) and is a more potent activator of membrane-recruited Raf-1 than H-Ras (Yan *et al.*, 1998). H-Ras activates PI3K more potently than K-Ras (Yan *et al.*, 1998). Enhanced motility induced by H-Ras (Kim *et al.*, 2003) suggested that H-Ras might be a more effective activator of the Rac pathway compared to N-Ras in MCF10A cells. Consistently, marked activation of Rac-MKK3/6-p38 pathway was exerted by H-Ras, but not by N-Ras, in MCF10A cells while the Raf-MEK-ERKs and PI3K-Akt pathways were activated by both H-Ras and N-Ras (Shin *et al.*, 2005).

(4) Ras signaling in invasion

Elevated levels of the Ras protein have been found in 60-70% of human primary breast carcinomas (Clair *et al.*, 1987), although Ras mutations are infrequent in human breast cancer. Ras expression has been suggested as a marker for tumor aggressiveness of breast cancer, including the degrees of invasion to fat tissues, infiltration into lymphatic vessels, and tumor recurrence (Clair *et al.*, 1987; Watson *et al.*, 1991; Clark and Der, 1995). The activated Ras proteins contribute to malignant phenotypes including invasiveness and angiogenesis (Shields *et al.*, 2000; Downward, 2003).

Recent reports investigated the role of Ras and Ras-dependent signaling pathways in cell invasion and migration. The Ras-PI3K-Akt pathway, which induces invasion and metastasis, downregulates RhoB, a suppressor of transformation, invasion and metastasis (Jiang *et al.*, 2004). The Rac-MKK3/6-p38 pathway, activated by H-Ras, but not by N-Ras, is critical to the H-Ras-induced invasive and migrative phenotypes in MCF10A breast epithelial cells (Kim *et al.*, 2003; Shin *et al.*, 2005). Anti-migratory and anti-invasive effect of somatostatin involves Rac, PI3K, and ERKs pathways in human neuroblastoma cells (Pola *et al.*, 2003). The Ras-MEKK1 pathway mediates lysophosphatidic acid-induced ovarian cancer cell migration (Bian *et al.*, 2004).

An essential part of the metastatic process includes degradation of the basement membrane and the stromal extracellular matrix, which allows breast cells to migrate into neighboring tissues. Members of matrix metalloproteinase (MMP) family, especially, MMP-2 (72 kDa type IV collagenase, gelatinase A) and MMP-9 (92 kDa type IV collagenase, gelatinase B), have been shown to be deeply involved in tumor invasion and metastasis formation (Ura *et al.*, 1989; Stetler-Stevenson, 1990; Liotta *et al.*, 1991;

Tryggvason, 1993). In rat and human embryonic fibroblasts, H-Ras mediated transformation and invasiveness were shown to be associated with enhanced expression of MMP-9 mRNA and protein (Bernhard *et al.*, 1990, 1994). In human breast epithelial cells, however, H-Ras-induced invasive phenotype is associated more closely with the expression of MMP-2 rather than MMP-9 (Moon *et al.*, 2000), demonstrating that Ras-mediated cellular responses differ between epithelial cells and fibroblasts (Oldham *et al.*, 1996). A direct correlation between high level of MMP-2 expression and an increased invasive capacity of tumor cell lines has been demonstrated both *in vitro* and *in vivo* (Sato *et al.*, 1992; Stetler-Stevenson *et al.*, 1996; Bodey *et al.*, 2001). Mounting evidence demonstrates a role for MMP-2 in the invasion of breast cancer cells and risk for a relapse in breast cancer patients (Moon *et al.*, 2000; Talvensaari-Mattila *et al.*, 2001; Nakopoulou *et al.*, 2003).

Ras signaling pathways responsible for regulating up-regulation of MMP-2 and MMP-9 causing invasive phenotype have been elucidated. The role of ERKs pathway in regulation of MMP-9 and invasion was demonstrated (Simon *et al.*, 1996; Gum *et al.*, 1997). The p38 pathway was also shown to regulate MMP-9 expression and *in vitro* invasion (Simon *et al.*, 1998; Simon *et al.*, 2001). Recent reports show that induction of MMP-9 expression is mediated by Ras-ERK and PI3K-Akt pathways (Chung *et al.*, 2004; Moon *et al.*, 2004). Although the signaling pathways regulating MMP-2 expression have been relatively poorly elucidated thus far, a recent study showed that insulin-like growth factor-I up-regulated MMP-2 expression *via* PI3K-Akt signaling while concomitantly transmitting a negative regulatory signal *via* the Raf-ERK pathway in lung carcinoma cells (Zhang *et al.*, 2004). Activation of Rac, PI3K, ERKs and p38 is crucial for H-Ras-induced up-regulation of MMP-2 and MMP-9 which play key roles in invasion and migration in MCF10A cells (Kim *et al.*, 2003; Shin *et al.*, 2005). The MKK6-p38 pathway alone was able to induce MMP-2 expression as well as invasive/migrative phenotype in MCF10A cells (Shin *et al.*, 2005). Activation of p38 pathway did not induce MMP-9 expression, suggesting that MMP-2 up-regulation by MKK3-p38 signaling plays a key role in invasion of breast epithelial cells. Based on the knowledge obtained in the MCF10A human breast epithelial cell system, a working model was proposed for the differential regulation of signaling pathways by H-Ras and N-Ras leading to proliferation, anchorage-independent growth, invasion and migration (Fig. 2).

Although MMP-2 and MMP-9 share structural and catalytic similarities, previous studies suggest that transcription of MMP-2 and MMP-9 may be independently regulated due to distinct arrays of *cis*-acting elements in the promoter. While the regulation of MMP-9 gene expression has been extensively studied (Sato, 1993; Sato and Seiki, 1993), the molecular basis of the regulating mechanisms for MMP-2 expression has not been well identified at the transcriptional level. AP-1 was demonstrated to play a crucial role in tumorigenesis especially in breast cancer (Li *et al.*, 1997, 1998, 2000; Ludes-Meyers *et al.*, 2001). Although MMP-2 was considered to be an AP-1-unresponsive gene in many cell types (Brown *et al.*, 1990; Tryggvason *et al.*, 1990; Qin *et al.*, 1999), recent data indicate that a functional AP-1 site mediates MMP-2 transcription in cardiac cells and breast cancer cells (Bergman *et al.*, 2003; Bachmeier *et al.*, 2005). The putative binding elements for p53, AP-1, Ets-1, C/EBP, CREB, PEA3, Sp1, and AP-2 have been found in the region of MMP-2 promoter (Bian and Sun, 1997; Qin *et*

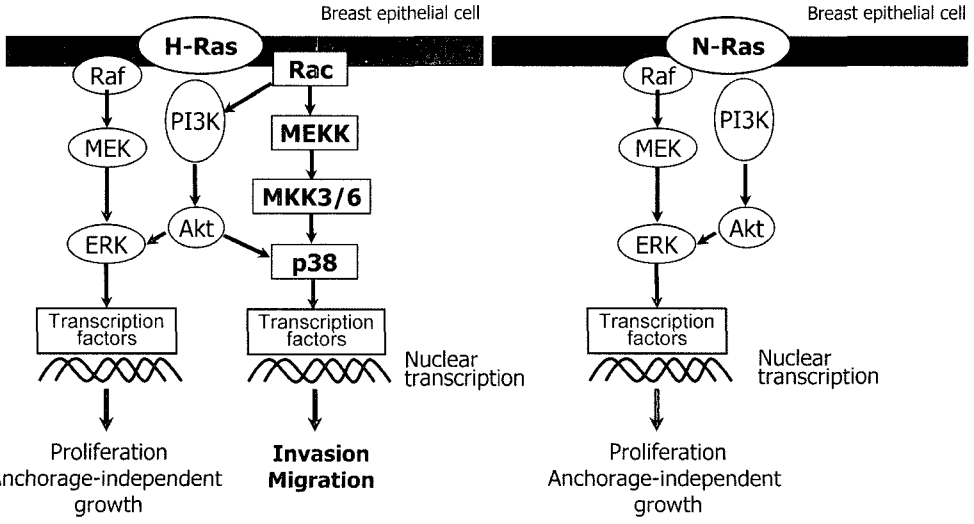

Fig. 2. Signaling pathways activated by H-Ras and N-Ras leading to proliferation and malignant phenotypic conversion in breast epithelial cell system

al., 1999; Bergman *et al.*, 2003). Given that MMP-2 has been suggested as a key factor responsible for H-Ras-induced invasion and migration of breast epithelial cells, further studies need to be performed to characterize which elements are functionally active in the transcriptional activation of MMP-2 gene leading to the malignant phenotypic conversion of breast epithelial cells.

CONCLUSIONS

For over 20 years since Ras was discovered, an enormous amount of knowledge has been accumulated as to how the Ras proteins function. The signaling network they regulate is very complex with multiple functions and cross-talks. The review summarizes some of the current understanding of Ras signaling with a major focus in its contribution to the invasive phenotypic conversion of epithelial cells. Four issues have been addressed in this review. First, Ras family consists of three members: H-Ras, K-Ras (A and B), and N-Ras. The greatest sequence divergence is seen in the C-terminal 25 residues, HVR, which is therefore potentially responsible for the distinct function of each isoform of Ras. Secondly, it is important to identify a differential membrane localization of Ras isofoms since a proper localization to the plama membrane is essential for the biological activity. HVR contains sequences for membrane anchoring of Ras including CAAX motif and lipidated residues. Detailed comparison of microlocalization of Ras isoforms needs to be performed. Thirdly, Ras proteins transduce extracellular signals to diverse cellular functions by stimulating multiple signaling networks. Utilization of the distinct sets of multiple signal transduction pathways regulated by Ras proteins accounts for the differential functions of H-Ras, K-Ras, and N-Ras in cell systems. Lastly, this review summarized recent studies on the differential role of Ras proteins in regulating signal molecules leading to invasion and migration with an emphasis on the Ras-induced up-regulation of MMP-2 and MMP-9. Although much has been known on the differential functions of Ras proteins, much more remains to be elucidated to provide implications on detailed understanding of molecular events for malignant phenotypic conversion of breast cells induced by Ras proteins.

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