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Ras signal transduction in carcinogenesis and progression of bladder cancer: molecular target for treatment?

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Abstract *Ras* oncogenes are considered to play a key role in the carcinogenesis and progression of human bladder cancer. The oncogenes code for the Ras p21 proteins, which localize in the internal part of the cell membrane and act as molecular switches to mediate downstream signaling from a variety of extracellular stimuli. Activation of Ras proteins induces the constitutive activation of downstream kinase cascades, which results in continuous mitogenic signaling and transformation of immortalized cells in human bladder cancer. Therefore inactivation of the activated Ras function might be effective for the development of a novel treatment strategy against human bladder cancer. Recently several ways to suppress Ras activities, including inhibitors of Ras signal transduction and a *ras* suppressor mutant, have been reported. Here we review the current concepts of the basic mechanisms of the intracellular Ras signaling pathway and *ras* activation in the carcinogenesis and progression of human bladder cancer and discuss clinical potentials of their therapeutic interventions.

Keywords Bladder cancer · Carcinogenesis · Progression · Intracellular signaling pathway · *Ras* oncogene

Introduction

The carcinogenesis of human bladder cancer is a multistep process. Several molecular alterations in oncogenes and tumor suppressor genes have been reported in human bladder cancers [15, 41, 56, 82]. Among these, *ras* oncogenes are considered to play a key role in the carcinogenesis of human bladder cancer [22, 33, 47]. The *ras* oncogenes (H-, K- and N-*ras*) code for the same product, designated p21 [36, 50, 55]. This product denotes a group of structurally and immunologically related proteins, with a molecular weight of 21 kDa, localized in the internal part of the cell membrane and with a function similar to that of G protein. The *ras* oncogene is frequently found to be activated in human malignancies [9, 58, 65, 88]. Single amino acid substitutions at codon 12, 13, or 61 of Ras protein result in aberrant Ras proteins that contribute to the formation of malignancy due to the disruption of normal GTPase activity of the protein [5, 17, 27, 52]. Once *ras* genes are activated in such a way, activated Ras proteins induce the constitutive activation of downstream kinase cascades, which results in a continuous mitogenic signaling and transformation of immortalized cells [11, 13, 31, 44]. Although activation of the *ras* gene might be therefore important in not only carcinogenesis but also the maintenance of solid tumors [14], its precise role in the pathogenesis of bladder cancer is still controversial. Questions remain as to whether only *ras* mutation can induce bladder tumorigenesis, why the frequency of *ras* mutations in bladder cancer ranged from 6–84% in previous reports [71, 72], and why the frequency of *ras* mutation is not always correlated to tumor stage [22, 32, 72]. Since multiple genetic changes occur simultaneously in human bladder cancer, the role of a single genetic alteration in inducing urothelial carcinogenesis has been difficult to clarify. Zhang and associates [96] provided direct experimental evidence that the urothelial expression of an activated H-*ras* in transgenic mice induced urothelial hyperplasia and superficial papillary bladder cancer, indicating that *ras* activation exerts a potent, proliferative effect on urothelial cells. Moreover, since Ras proteins are key molecules in signal transduction of cellular proliferation [5, 37], mechanisms of *ras* gene activation other than point mutation might be involved in the carcinogenesis of bladder cancer. Recently several intracellular signal

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transduction pathways and signaling networks that are activated by cell surface receptors with intrinsic tyrosine kinase activity have been extensively investigated [77]. Growth factor binding to receptor tyrosine kinase (RTK), including that by members of the epidermal growth factor (EGF)/transforming growth factor (TGF)α and fibroblast growth factor families, leads to receptor dimerization, activation of kinase activity and autophosphorylation on tyrosine residues. The adaptor protein, growth-factor receptor binding protein 2 (Grb2), forms a complex with son of sevenless (Sos), Ras guanine nucleotide exchange protein. The Grb2-Sos complex is recruited to activated RTK through binding to specific sites of the receptor, thus translocating Sos to the membrane. The Sos protein recruited to the membrane can interact with membrane-anchored Ras, converting Ras to the active GTP form [6, 76, 77]. Therefore upregulation of the Grb2-Sos complex might result in *ras* activation. Daly and associates [24] in fact reported that Grb2 protein and Sos protein were markedly overexpressed in some breast cancer cell lines relative to normal breast epithelial cells. We also showed evidence that Grb2 and Sos proteins were also overexpressed in human bladder cancer cell lines regardless of *ras* mutation [94].

This review article summarizes the basic mechanisms of the Ras signaling pathway and *ras* activation and discusses their clinical potential for therapeutic intervention in human bladder cancer.

Ras signaling pathway

Ras proteins are GDP/GTP binding proteins that function as molecular switches to mediate downstream signaling from a variety of extracellular stimuli that influence cellular proliferation and differentiation [74]. The Ras protein is initially synthesized as a cytoplasmic precursor which needs posttranslational processing to attain biological activity [34, 52]. The critical biochemical change that activates the Ras precursor is farnesylation of cysteine residues present in the Caax motif (C: cysteine; a: an aliphatic amino acid; X: another amino acid) located at the carboxyl-termini of Ras proteins. Once farnesylated and modified, the mature Ras protein is inserted into the plasma membrane where it participates in the signal transduction pathways from extracellular stimulation. The farnesylation reaction that modifies Ras proteins having an appropriate CaaX motif is catalyzed by farnesyltransferase using farnesyl diphosphate as a cosubstrate [34].

The Ras proteins are subsequently activated when the protein binds GTP and becomes inactive upon GTP hydrolysis to GDP by Ras proteins. The action of Ras proteins is regulated by several guanine-nucleotide exchange factors and GTPase-activating proteins (GAPs) [10]. Guanine-nucleotide exchange factors are localized at the membrane by the tyrosine kinase receptor, and interact with Ras proteins, forcing the release of GDP, allowing entry of another GTP molecule, and converting the active conformation of Ras proteins [7, 10]. Thus these factors result in positive regulation of *ras* activity. On the other hand, GAPs stimulate the intrinsic GTPase activity of Ras protein to promote formation of its inactive GDP-bound form [7, 10]. Since Ras-mediated signals are generated by the conformational change associated with the presence of GTP, GAPs may negatively regulate the activity of the normal Ras proteins by limiting the duration of signaling. Consequently, deregulated function of Ras guanine-nucleotide exchange factors and GTPase-activating proteins can also induce constitutive activation of Ras proteins and transformation of cells.

The mechanism of *ras* activation by the EGF receptor has recently been delineated [6, 76, 77] (Fig. 1). EGF binding to EGF receptor tyrosine kinases leads to receptor dimerization, activation of kinase activity, and autophosphorylation on tyrosine residues, thus creating specific binding sites for the adaptor protein, Grb2, containing src homology 2 domains [83]. The Grb2 protein also binds proline-rich motifs in the COOH terminus of the Ras guanine-nucleotide exchange factor through the src homology 3 domain [66]. Recent genetic studies have led to the identification of several Ras guanine-nucleotide exchange factors such as p140 Ras-GRF (CDC25 Mm) [81] and Sos protein [13]. Unlike p140 Ras-GRF, which is found only in the brain, Sos proteins are known to be expressed ubiquitously [11]. After the formation of a ternary complex containing Sos-Grb2-EGF tyrosine kinase receptors stimulated by EGF, Sos protein can increase the active GTP-bound form of Ras protein by promoting the guanine-nucleotide exchange reaction [3, 11]. Therefore even normal

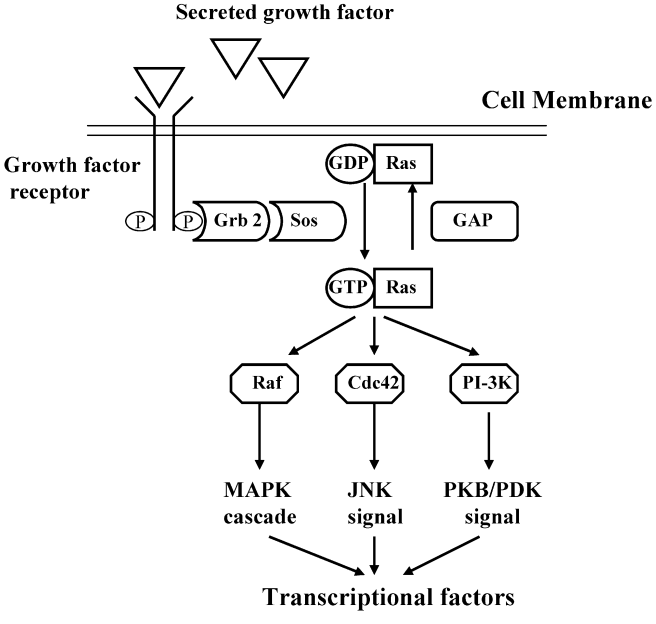

Fig. 1. Signaling pathway activated by receptor tyrosine kinase

EGF-associated growth stimulation may significantly enhance the cellular *ras* activity and consequently facilitate the proliferation of cells.

The active, GTP-bound form of Ras can interact with several effecter proteins, Raf serine/threonine kinase, Cdc42, and phospholipid kinase phosphatidylinositol 3 kinase to stimulate numerous intracellular processes [77]. Once activated by its interaction with Ras, Raf proteins can directly phospholylate the mitogen-activated protein kinase kinases (MAPKK or MEKs), generating their active or signaling forms in the cascade [91, 92] (Fig. 2). The MAPKKs in turn activate members of the mitogen-activated protein kinase (MAPK) family, ERK1 or ERK2 by phosphorylation of specific tyrosine and threonine residues [30, 61]. When activated, the MAPKs may directly phosphorylate certain nuclear transcription factors, or they may interact with other nuclear kinases, which in turn can phosphorylate transcription factors [38]. The MAPK cascade provides one of the central intracellular signaling pathways that control cell proliferation, cell differentiation, and early embryonic development.

Recent reports have indicated that activated Ras also causes activation of a Raf-independent pathway that leads to activation of the Jun-NH2 kinase (JNK) [25, 26, 42]. JNK activity requires mitogen-activated protein kinases kinases (MEKK) 1–4 which phosphorylates MKK4/7. MKK4/7 in turn phosphorylates JNK on residues 183 and 185 [25, 59]. Activated JNK phosphorylates its substrates, c-Jun, ATF2, and ELK1 [25, 48]. Clark and associates [18] reported that inhibition of JNK activation inhibited Ras transformation of NIH3T3 cells. Ras suppressor Rsu-1 suppressed Ras transformation by inhibition of JNK activation even though the Raf-mediating pathway was enhanced [54]. Furthermore, Watanabe and associates [93] showed that suppression of transformed phenotypes could be brought about by the inhibition of JNK phosphorylation in a human bladder cancer cell line with a mutated *ras* oncogene. These results suggest that JNK activation via a Raf-independent pathway is also associated with Ras-mediated transformation.

In this manner the actual mechanisms of the Ras-signaling pathway are considered to be more complex than presented until now [25, 76, 77]. Furthermore, cell type differences exist regarding the signaling pathways that promote Ras activation as shown by Platter and associates [68]. However, since several growth factors signal through the same general mechanism, Ras-mediated signaling could play an important role in the control of several intracellular processes such as metabolism, cell cycle, cell shape, proliferation, and differentiation.

Activation of *ras* oncogene and its significance in bladder cancer

The association of the mutated *ras* gene family (H-, K-, and N-*ras*) with 30% of all human cancers suggests an important contribution of aberrant Ras function to the development of human cancers [5, 9, 17]. Frequently mutated "hot spots" are glycine to valine in codon 12, glycine to cysteine at codon 13, and glutamine to arginine/lysine/leucine at codon 61 [8, 49]. Point mutations in *ras* genes block the intrinsic GTPase activity, thus preventing the normal deactivation of Ras proteins [5, 17, 27, 52]. The incidence of *ras* mutation varies greatly depending on the tissue or cell type from which cancer cells are derived, although *ras* mutation occurs in 75–95% of pancreatic carcinomas [2, 85] and 50% of colon carcinomas [90], it is rare in several tumor types. The H-*ras* mutation was first detected in the human bladder cancer cell line T24 [12] (Table 1). Subsequent studies demonstrated that H-*ras* mutations are more frequently detected in urinary tract tumors than are the K-*ras* or N-*ras* genes [72]. The initial expectation that *ras* mutation might play an important role in bladder carcinogenesis has decreased, since analyses of uncultured bladder tumors have shown that only about 10% contain a mutated H-*ras* gene [33, 46, 73]. However, more recent reports showed a significantly higher frequency of H-*ras* mutations in bladder tumors. Fitzgerald and associates [32] reported that mutations in the H-*ras* gene

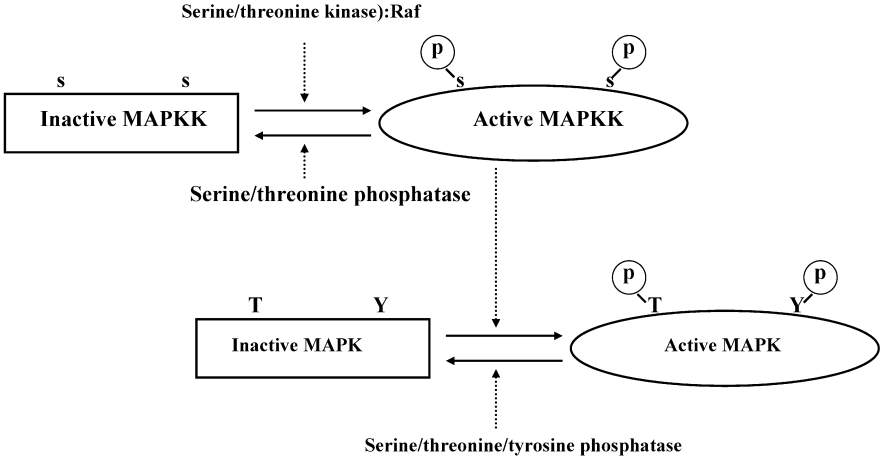

Fig. 2. MAPK signaling cascade and mechanism for its control

Table 1. *ras* activation in human bladder cancer

Mechanism	Incident rate	References
Point mutation of *ras* gene	6–84%	22, 32, 33, 46, 71, 72, 73
Overexpression of Ras protein	40–50%	88, 89
Functional activation of Ras signaling pathway	Unknown	93, 94

were detected in urine sediments from 44% of bladder cancer patients. Czerniak and associates [22] observed H-*ras* mutation at codon 12 in 45% of bladder cancers. Furthermore, Przybojewska and associates [71] found the H-*ras* mutation in 84% of bladder cancer patients by using a polymerase chain reaction-restriction fragment length polymorphism assay. The discrepancies may be due to the method used for the detection of *ras* mutation, and the precise frequency of *ras* mutation in human bladder cancer is still unclear. An alternative mechanism of *ras* gene activation in human bladder cancer is quantitative alterations in expression due to gene amplification, thus maintaining continuous proliferative signals. Viola and associates [89] found increased expression of Ras protein in carcinoma in situ and high-grade tumors but not in hyperplasia or low-grade tumors by immunohistochemical study. Vageli and associates [88] also reported that the ras transcripts were increased in about 40% of bladder cancer specimens. Although these results still remain controversial, activation of the *ras* oncogene by point mutation or overexpression might be important in the carcinogenesis and progression of human bladder cancer.

More recently, functional activation of the Ras signaling pathway has been shown as new mechanism for *ras* activation. Several extracellular growth signals such EGF and fibroblast growth factor function through the Ras signaling pathway as discussed previously. Since the Sos protein can increase the active GTP-bound form of Ras protein by promoting the guanine-nucleotide exchange reaction, qualitative and/or quantitative change in growth factor receptor, Grb2 protein and Sos protein could bring about functional *ras* activation and promotion of cellular proliferation and transformation. Guha and associates [35] showed that, although oncogenic *ras* mutations are not prevalent in human malignant astrocytomas, levels of the GTP-bound form of Ras proteins were elevated in these cells secondary to the mitogenic signals originating from activated receptor tyrosine kinases. In human bladder cancer upregulation of several growth factor receptors has also been shown to be associated with carcinogenesis and progression [51]. Of these, the EGF receptor has been widely analyzed and is considered to play a key role in human bladder cancer. EGF is a specific ligand of the EGF receptor and is produced in a variety of tissues [53]. EGF is present in large amounts in urine [23], and some of this urinary EGF might act as a ligand of EGF receptors and function as an extracellular growth signal in bladder cancer. Overexpression of EGF receptors is observed in some bladder cancers, with increasing expression being seen with increasing stage and pathological grade [56, 60]. Overexpressed EGF receptors can bind EGF, are phosphorylated, and thus induce increased tumor proliferation in human bladder cancer [75, 86]. However, these studies did not answer the question of whether several factors related to the signals downstream from the EGF receptor are enhanced in bladder cancer.

Recently Daly and associates [24] reported that Grb2 protein was markedly overexpressed in some breast cancer cell lines as compared with normal breast epithelial cells, suggesting that this up-regulation of the Ras signaling pathway might modulate the growth-factor sensitivity of these cell lines and therefore play a role in tumor progression. We also demonstrated that there was overexpression of Sos protein in human renal cell carcinoma cell lines [80]. Furthermore, Watanabe and associates [94] examined expression of the EGF-receptor, Grb2, and Sos protein in five human bladder cancer cell lines and two cultured normal urothelial cells. Although no significant difference in expression of the EGF receptor was observed among these cells, the expression of Grb2 and Sos proteins was enhanced in all human bladder cancer cell lines examined compared with normal urothelial cells. These results suggest the possibility that the expression of Grb2 and Sos proteins is more critical than expression of the EGF receptor for the change from normal urothelial cells to bladder cancer cells. The EGF-triggered signaling might not be enhanced in normal urothelial cells because of the low expression levels of Grb2 and Sos as compared to human bladder cancer cells.

Potential for therapeutic intervention

As shown above, aberrant cellular Ras function induced by activating mutation, overexpression, and functional activation of the signaling pathway is important for the formation of bladder cancer. Inactivation of the activated Ras function might therefore be effective for the development of novel treatment strategies against human bladder cancer. Many ways to control the activity of Ras for the regression of tumors have been suggested, including an inhibitor of Ras signal transduction, neutralizing anti-Ras monoclonal antibodies [19, 87], an anti-Ras ribozyme [29], antisense oligonucleotides of the *ras* gene [45, 75], and a *ras* suppressor mutant [62, 63, 64] (Fig. 3).

Farnesyltransferase inhibitor

With regard to inhibitors of ras activation, farnesyltransferase inhibitor (FTI) has been well investigated [34, 43, 52]. FTIs represent a new class of agents that target signal transduction pathways responsible for the

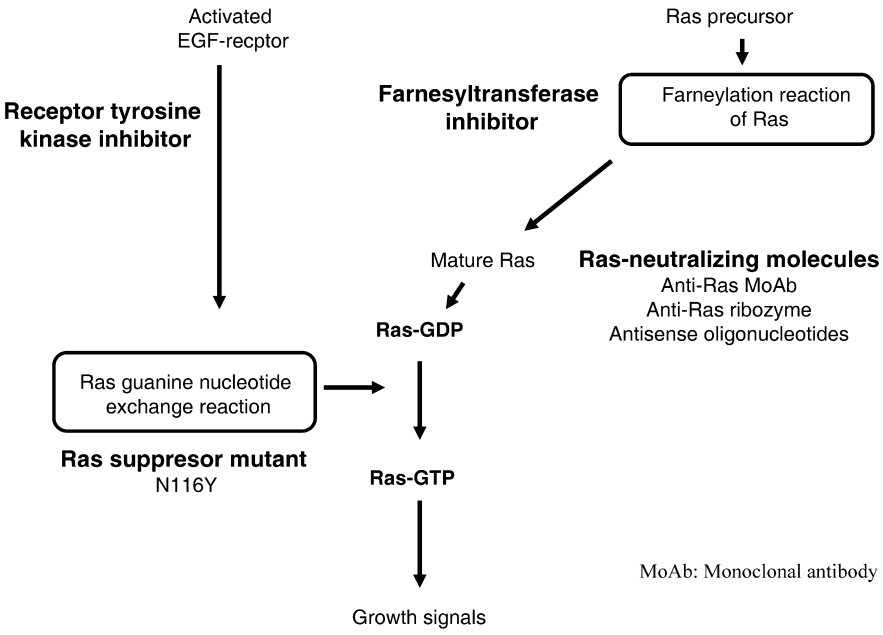

Fig. 3. Mechanisms and sites of action of therapeutic intervention on the Ras signaling pathway

proliferation and survival of diverse malignant cell types. These agents were developed to prevent a processing step necessary for membrane attachment and maturation of Ras proteins. To date several FTIs have been developed as anticancer drugs: SCH66336, R115777, L744832, and BIM-46228 [1, 21, 69, 70, 97]. Clinical trials of two FTIs, SCH66336 and R115777, have demonstrated disease stabilization or objective responses in 10–15% of patients with refractory malignancies [1, 69]. Although no clinical trial on the clinical efficacy of FTIs against human bladder cancer has been conducted, such trials should be performed since *ras* activation plays a key role in the progression of human bladder cancer.

Receptor tyrosine kinase inhibitor

More recently several receptor tyrosine kinase inhibitors have been developed for novel treatment of human malignancies [4, 16, 28, 40]. These drugs have been used in clinical trials and shown to have antitumor effects. The EGF receptor tyrosine kinase inhibitors ZD-1839 and OSI-774 are widely recognized to have significant antitumor activities, even used as single agents [4, 16]. Meye and associates [57] reported that ZD-1839 inhibited the EGF-stimulated proliferation in dose-dependent manner and increased apoptosis in some human cancer cell lines. Furthermore, combined treatment with ZD-1839 and a cytotoxic agent, such as cisplatin and paclitaxel, produced tumor growth inhibition in several human cancer cell lines, providing a rationale for its clinical evaluation in combination with cytotoxic drugs [16, 83]. These results also highlight the potential clinical benefit of ZD-1839 alone or combined with cytotoxic agents in the treatment of human bladder cancer since the expression of EGF receptor and EGF-triggered downstream signals have been shown to be up-regulated.

Ras-neutralizing molecules

Another way for controlling and inhibiting Ras activity is the use of the Ras-neutralizing molecules [19, 87]. Cochet and associates [19] prepared a single-chain Fv fragment (scFv) derived from the neutralizing anti-Ras monoclonal antibody Y13-239 and showed that this neutralizing Ras specifically promoted apoptosis in vitro in human cancer cells but not in untransformed cells. They also demonstrated that intratumor transduction of HCT116 colon carcinoma cells with the anti-Ras scFv using an adenoviral vector elicited sustained tumor regression in nude mice. Kita and associates [45] investigated the inhibitory effects of antisense oligonucleotides targeting K-*ras* point mutation on the growth of cultured human pancreatic cancer cells with K-*ras* mutation. Their results showed that mutation-matched antisense oligonucleotides effectively inhibited the growth of the pancreatic cancer cell lines by suppressing K-*ras* mRNA expression and K-Ras p21 protein synthesis. Although these results are very interesting, no data on the impact of these neutralizing molecules on human bladder cancer cell lines have been presented.

Dominant negative H-*ras* mutant and gene therapy

The dominant negative H-*ras* mutant N116Y was derived from the v-H-*ras* oncogene by substitution of tyrosine for asparagine-116. This mutant has been shown to suppress the transformed phenotypes of NIH/3T3 cells induced by overexpression of the H-*ras*

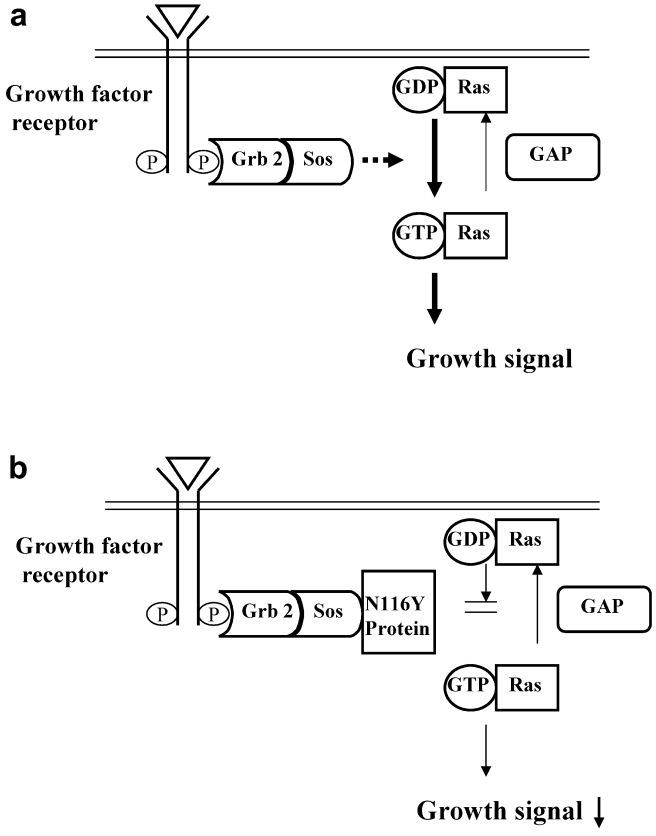

Fig. 4a, b. Hypothesis of N116Y action in signal transduction. **a** Growth signal by activated receptor tyrosine kinase. **b** Inhibition of growth signal by N116Y *ras* mutant

proto-oncogene and several protein tyrosine kinase oncogenes [62, 63]. The N116Y *ras* mutant is considered to prevent the GTP-form of endogenous Ras by inhibiting the Ras guanine nucleotide exchange reaction [39] (Fig. 4). The N116Y *ras* mutant also significantly inhibited the growth of a variety of human cancer cells, including human bladder cancer cell line T24 [64, 80]. N116Y could also suppress the transformed phenotype of bladder and pancreatic cancer cell lines, even if that expression was not strong enough to suppress growth [79, 93]. Furthermore, introduction of the N116Y *ras* mutant in vivo via an N116Y-containing adenoviral vector (AdCMV-N116Y) strongly suppressed in vivo growth of esophageal cancer cells [78]. Using this adenoviral vector system, we investigated the growth-suppressive effects of N116Y on orthotopically implanted bladder cancer cells in vivo [95]. The results demonstrated that inoculation of AdCMV-N116Y caused significant growth suppression of orthotopically implanted bladder cancer cells. Although it is still in the preclinical stage, gene therapy via transurethral inoculation of AdCMV-N116Y might hold promise for the treatment of human bladder cancer.

At the present time the majority of treatments for advanced bladder cancers, such as systemic chemotherapy, immunotherapy, and radiation therapy, are not always effective [20, 67]. Attempts to explore novel therapeutic modalities are under way in the hope that understanding of the biology of this tumor will lead to means for improvement in the survival rate. Of these, molecular-target therapy, including gene therapy, for human cancers has lately attracted considerable attention. In particular, intervention in the Ras signaling pathway utilizing inhibitors such as the N116Y *ras* mutant and tyrosine kinase inhibitor might be one of the effective approaches. In the near future these modalities may play important roles in the treatment of human bladder cancer.

Conclusion

Considerable progress has been made toward elucidating the Ras signaling pathway in human malignancies, including bladder cancer. Although point mutation of the *ras* proto-oncogene is important, quantitative and/or qualified changes in the upstream activators of Ras, the EGF-receptor, Grb2, and Sos proteins also appear to be associated with the carcinogenesis and progression of human bladder cancer. Therefore modulation of this signal transduction by *ras* suppressor mutant and several inhibitors would offer novel treatment modalities against human bladder cancer.

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