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Review

Carcinogenic Role of Tumor Necrosis Factor-α Inducing Protein of *Helicobacter pylori* in Human Stomach

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***Helicobacter pylori* is the definitive carcinogen for stomach cancer and is known to induce proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) in the stomach. Based on our findings that TNF-α is an endogenous tumor promoter, we identified the TNF-α inducing protein (Tipα) gene family, and confirmed Tipα and HP-MP1 as new carcinogenic proteins of *H. pylori*. Tipα protein is unique to *H. pylori*, and this paper shows the strong tumor promoting activity of Tipα gene family, in cooperation with Ras protein and its mechanisms of action in relation to NF-κB activation, and discusses the carcinogenic role of Tipα in stomach cancer. Our recent finding showing that penicillin-binding proteins of other bacteria are weak homologues of Tipα is also discussed.**

Keywords: NF-κB, Stomach, TNF-α, Tumor promotion

Introduction

In 1983, Barry Marshall and Robin Warren identified *Helicobacter pylori* as a bacterium closely associated with chronic gastritis and peptic ulcer. (Marshall and Warren, 1984). In 1994, the IARC (WHO) classified *H. pylori* infection as the definitive carcinogen for humans, based on epidemiological studies (IARC Working Group, 1994), and in 2005, Marshall and Warren were awarded the Nobel prize for Medicine and Physiology. *H. pylori* is a spiral-shaped, Gram-negative bacterium which attaches to gastric epithelial cells in the human stomach and infects about 50% of the world's population (Correa, 2003). Japan and Korea have the highest incidence rates of gastric cancer in the world, and the prevalence rate of *H. pylori* remains high - 80 to 90% - over the age of 40, although it has been decreasing (Lee *et al*., 2005; Penta *et al*., 2005). To develop a prevention and molecular targeted therapy for stomach cancer, the investigation of carcinogenic process in *H. pylori* infection is a key point for both nations. Various virulence factors of *H. pylori*, such as cytotoxin-associated gene Pathogenicity Island (*cag* PAI), cagA, vacuolating cytotoxin A (vacA), and urease, have been studied, and the strong association of *cag* PAI with the occurrence of peptic ulcers and cancer has been reported (Atherton, *et al*., 1995, Censini *et al*., 1996; Covacci *at al*., 1999; Peek Jr. and Blaser, 2002; Normark *et al*., 2003). However, the high frequency of *cag* PAI$^+$ *H. pylor* - nearly 100% in clinical isolates in Japan and Korea - indicates the contribution of additional virulence factors for cancer development (Shimoyama *et al*., 1997; Park *et al*., 1998; Covacci *at al*., 1999).

It is well known that *H. pylori* infection induces inflammation in microenvironment of the stomach associated with induction of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6 and IL-8, based on the results showing that the mucosal levels of these cytokines are significantly higher in *H. pylori* positive patients than in negative patients (Crabtree *et al*., 1991; Noach *et al*., 1994). Urease, cagA and *Helicobacter pylori*-membrane protein-1 (HP-MP1) of *H. pylori* induce TNF-α in human cells (Harris *et al*. 1996; Yamaoka *et al*., 1997; Yoshida *et al*., 1999). But precisely how proinflammatory cytokines induced by *H. pylori* infection are involved in stomach cancer is not well understood. From our long-time investigation on the mechanisms of tumor promotion, we looked again at that TNF-α is the key cytokine in tumor promotion, even though TNF-α was originally identified as a serum factor inducing hemorrhagic necrosis of transplanted solid tumors in mice (Old, 1985; Komori, *et al*., 1993; Fujiki *et al*., 2002; Fujiki and Suganuma, 2005). Our findings are: 1) TNF-α gene is commonly induced by tumor promoters, such as 12-*O*-tetradecanoylpholbol-13-acetate (TPA), okadaic acid, microcystin and nodularin, in various target organs, such as

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mouse skin, rat glandular stomach and rat liver, and TNF-α pathway is a common mechanism of tumor promotion (Fujiki and Suganuma, 1993). 2) TNF-α itself is a strong tumor promoter, inducing *in vitro* transformation of BALB/3T3 cells (Komori *et al*., 1993). 3) TNF-α-deficient mice were refractory to tumor promotion on mouse skin with TPA and okadaic acid in two-stage carcinogenesis experiments (Moore *et al*., 1999; Suganuma *et al*. 1999). 4) The TNF-α of tumor promotion begins from TNF-α as the first instigator through IL-1 and IL-6 (Suganuma *et al*., 2002). Independently, Tatematsu and his associates reported that *H. pylori* infection induces tumor promotion in Mongolian gerbil stomach initiated with various carcinogens (Sugiyama *et al*., 1998; Shimizu *et al*., 1999). Taken together, we hypothesized that *H. pylori* gene products must have TNF-α inducing activity and act as tumor promoters in stomach carcinogenesis. We found that the proteins of the TNF-α inducing protein (Tipα) gene family in *H. pylori* genome are Tipα and HP-MP1, and that they act as new carcinogenic factors of *H. pylori* mediated through strong induction of TNF-α gene expression and nuclear factor-κB (NF-κB) activation (Suganuma *et al*., 2001; Suganuma *et al*., 2005). This article reviews the proteins of Tipα gene family and discusses their mechanisms of action in stomach carcinogenesis. Recently, we extended the concept of Tipα gene family to genomes of other bacteria: These new results are also included in the text.

TNF-α inducing protein (Tipα) gene family

The products of Tipα gene family are defined as *H. pylori* proteins that strongly induce TNF-α gene expression and also possess *in vitro* transforming activity. Those identified so far are Tipα, HP-MP1 and probably jhp0543 of strain J99 (Alm *et al*., 1999; Suganuma *et al*., 2005). HP-MP1 gene was first cloned from genomic DNA of *H. pylori* strain SR7791 as an antigenic membrane protein with a potential for inducing production of TNF-α, IL-1α and IL-8 in human monocytes (Yoshida *et al*., 1999). We also identified HP0596 gene of *H. pylori* as a TNF-α inducing protein (Tipα) gene from genomic sequence of *H. pylori* strain 26695, which is homologous to HP-MP1 gene with 94.3% homology (Tomb *et al*., 1997; Suganuma *et al*., 2005). However, we found that HP0596 protein was released from *H. pylori* into culture broth, so we named the HP0596 gene a (Tipα) gene in the functional sense. Tipα gene family is not in *cag* PAI region, and the gene has no sequence similarity to cagA, vacA, or urease gene. Furthermore, there is no obvious homolog among other species, indicating that Tipα gene family is a unique gene for *H. pylori*.

The deduced amino acid sequence of Tipα gene product was revealed to be a protein of 192 amino acids with 21.8 kDa, to have a signal sequence in the N-terminal region of 20 amino acids and to have 94.8% identity between Tipα and HP-MP1 proteins (Fig. 1). Therefore, we call the proteins of this gene family, Tipα. Tipα protein is present in various strains, 26695, *cag* PAI deletion mutant (26695Δ*cag* PAI), ATCC43504, and SS1, and moreover, it has been found in clinical isolates obtained from patients with ailments such as gastritis, gastric ulcer, duodenal ulcer and gastric cancer (Suganuma *et al*., 2005). Western blot analysis of *H. pylori* extract using specific antibody against 19 mer oligopeptides (31 - 48 amino acid residues) showed 2 bands of 38 kDa and 19 kDa proteins in the absence of dithiothreitol (DTT), and

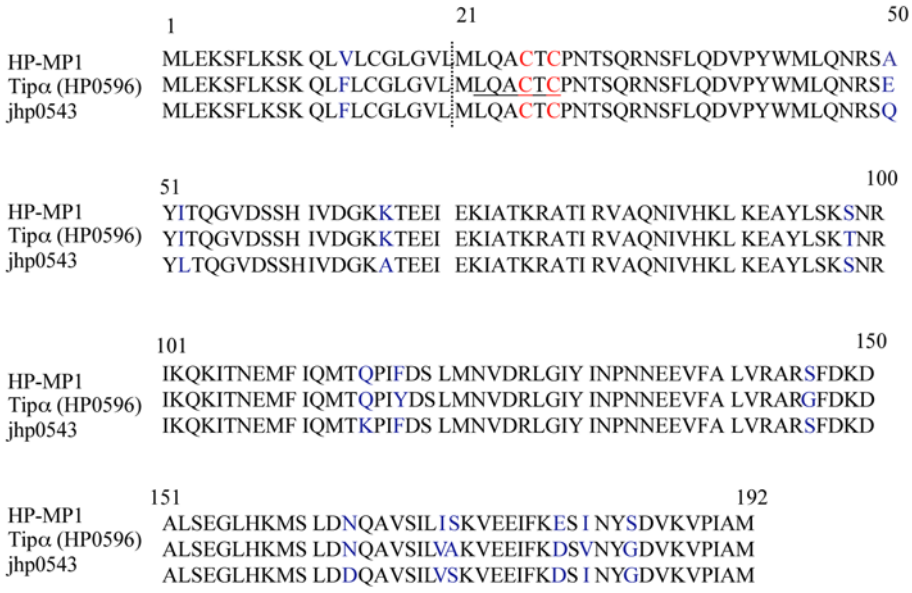

Fig. 1. The amino acid sequence of the proteins of Tipα gene family: HP-MP1, Tipα and jhp0543. There is 94.8% identity among the three. Blue characters indicate different amino acids among them, and a dotted line and underline indicate a cleavage site of Tipα and deleted 6 amino acids in rdel-Tipα, respectively.

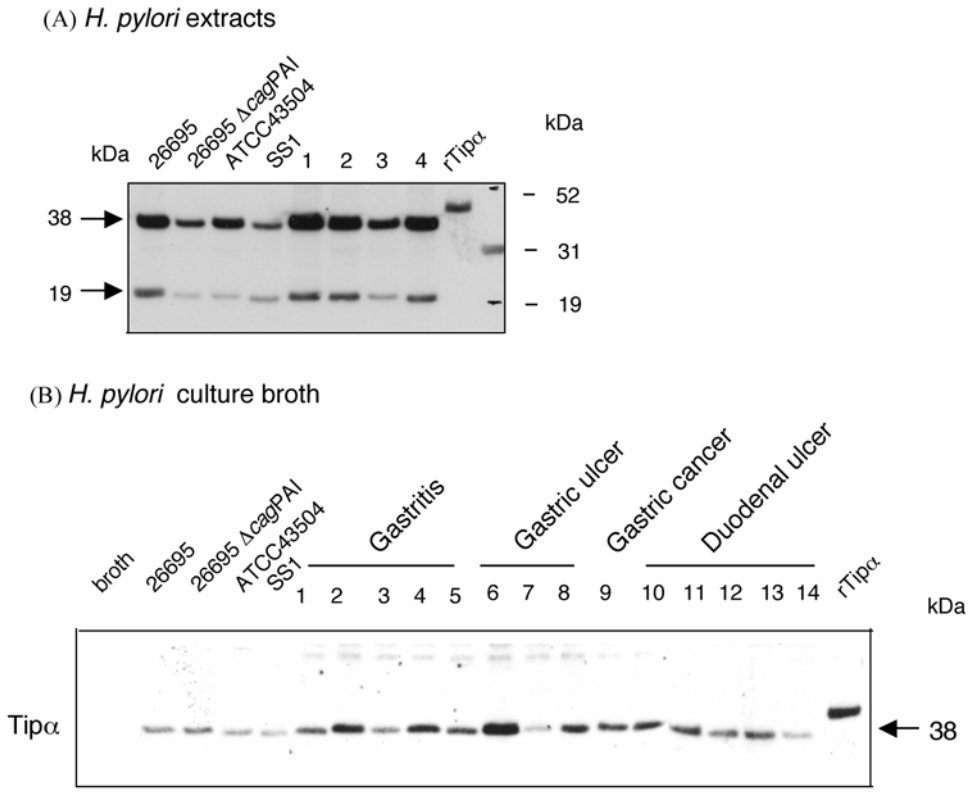

Fig. 2. Tipα protein in various *H. pylori* strains. (A) The presence of Tipα as a homodimer form in *H. pylori* strains and clinical isolates from patients with ailments such as gastritis (1), gastric ulcer (2), gastric cancer (3), and duodenal ulcer (4). (B) Tipα homodeimer protein released from various *H. pylori* into culture broth.

only one band of 19 kDa in the presence of DTT: This indicated that Tipα protein consisted of 192 amino acids and was cleaved between 20 and 21 amino acids, forming a homodimer in *H. pylori* (Fig. 2A). A dimer form of Tipα protein was released from various strains and the results were all confirmed by clinical isolates of *H. pylori* (Fig. 2B). The deletion mutant of *cag* PAI (26695Δ*cag* PAI) *H. pylori* also released Tipα protein into culture broth, similar to a wild 26695 strain, and we think that Tipα protein is released from *H. pylori* mediated through some system different from type IV secretion (Odenbreit *et al*., 2000). It was of great interest to note that released levels of Tipα protein in culture broth varied among 14 clinical isolates.

Transforming activity in cooperation with RAS protein

Bhas 42 cells, which are BALB/3T3 cells transfected with v-H-ras gene, can be used as a model of initiated cells to examine the tumor promoting activity of Tipα product. This experimental procedure is a practical and useful tool to demonstrate *in vitro* tumor promoting activity of proteins (Sasaki *et al*., 1990; Ohmori *et al*., 2004): We and other investigators have proved the tumor promoting activity of some proteins, such as hepatitis C virus core protein, the leukemia-related protein MTG8 (ETO), and an extract of *Staphylococcus aureus* (Sueoka *et al*., 1998; Tsuchihara *et al*., 1999; Fujiki *et al*., 2004). We first transfected HP-MP1 gene, urease B gene, or vector alone as control, into Bhas 42 cells, and established Bhas/mp1, Bhas/ure and Bhas/vec clones. All Bhas/mp1 clones significantly expressed TNF-α gene much more strongly than did either parental Bhas 42 cells, or Bhas/ure and Bhas/vec clones (Suganuma *et al*., 2001) (Fig. 3A). On the other hand, HP-MP1 and urease B genes were similarly transfected into BALB/3T3 cells (without v-H-ras gene), but their clones did not show any significant expression of TNF-α gene (Fig. 3B). Thus, HP-MP1 gene significantly induced TNF-α gene expression only in the presence of v-H-ras gene.

The malignancy of these clones was examined by subcutaneous implantation into nude mice, and by anchorage-independent growth in soft agar: All three examined Bhas/mp1 clones rapidly produced tumors in mice associated with strong angiogenesis within 20 days after implantation, as did many colonies in soft agar (Table 1). In contrast, only two Bhas/ure clones, which expressed TNF-α gene, produced small tumors in mice later, and small numbers in soft agar colonies. Due to the absence of TNF-α gene expression, BALB/mp1 clones did not significantly produce any soft agar

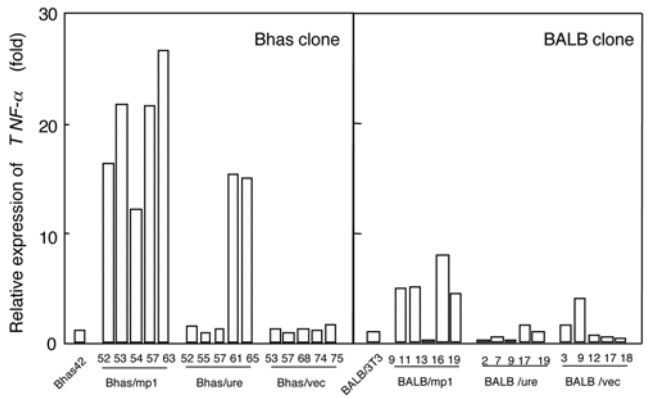

Fig. 3. Strong induction of TNF-α gene expression by transfection of HP-MP1 gene in cooperation with Ras protein. HP-MP1 gene, urease B gene or vector alone was transfected into Bhas 42 cells (with v-H-ras gene) and BALB/3T3 cells (without v-H-ras gene clones), and Bhas and BALB clones were obtained.

Table 1. Difference in carcinogenic potential between Bhas and BALB clones

Clones and cells	Tumorigenicity	
	Average no. of soft agar colonies	No. of sites with tumors/no. of injected sites
Bhas/mp1	20.0 ± 10.1	18/18 (100%)
Bhas/ure	4.1 ± 6.1	6/18 (33.3%)
Bhas/vec	2.3 ± 1.5	0/18 (0%)
Bhas 42	2.0 ±	0/6 (0%)
BALB/mp1	0.5 ± 0.9	ND[a]
BALB/ure	0.3 ± 0.4	ND
BALB/vec	0.2 ± 0.4	ND
BALB/3T3	0	ND

[a]ND, not determined.

colonies. These results clearly indicated the TNF-α inducing activity of HP-MP1 plays a significant role in carcinogenesis of *H. pylori* only in initiated cells. Transfected v-H-ras gene was used as initiation in this experiment, and the overexpression of H-, K-, and N-ras gene and Ras p21 proteins are often observed in human stomach cancer tissue and precursor lesions in stomach with *H. pylori* infection (Ohuchi *et al*., 1987; Wang *et al*., 2002). Taken together, we think that alteration of ras gene level is an initiation, and that *H. pylori* infection is the process of tumor promotion, i.e., a typical example of multi-step carcinogenesis in human stomach.

Tipα homodimer as an active form

To investigate the significance of released Tipα homodimer from *H. pylori* in carcinogenic activity, we made His-tagged recombinant Tipα protein (rTipα) consisting of 172 amino

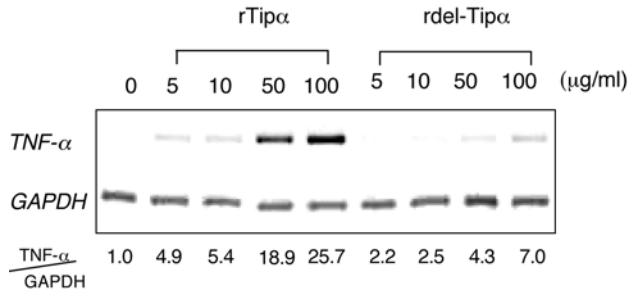

Fig. 4. Homodimer form of Tipα is an active form in TNF-α gene expression. Treatment with rTipα (a homodimer form) induced TNF-α gene expression in mouse epithelial cells (MGT-40), but rdel-Tipα (an inactive form) did not.

acids (from 21 to 192): rTipα protein forms a homodimer with a molecular weight of 42 kDa similar to native Tipα Suganuma *et al*., 2005). Since Tipα has only two cysteine residues, at positions 25 and 27, we made a deletion mutant of Tipα (rdel-Tipα) that lacked six amino acids (22-27 containing two cysteine residues) (Fig. 1). rDel-Tipα showed only a monomer form with 21 kDa, suggesting that these two cysteines contribute to the homodimer formation of Tipα by disulfide bonds.

Treatment with rTipα protein strongly induced TNF-α gene expression not only in Bhas 42 cells but also in mouse gastric epithelial cell line MGT-40 (Ichinose *et al*., 1998), indicating that Tipα protein has TNF-α inducing activity in gastric epithelial cells (Fig. 4). However, treatment with rdel-Tipα even at concentrations up to 100 μg/ml did not significantly enhance it in either cell line. As for the consequence of TNF-α-inducing activity, rTipα protein significantly induced transformation of Bhas 42 cells *in vitro*, but rdel-Tipα did not. Moreover, the tumor promoting activity of Tipα was quite similar to TPA, a potent tumor promoter: rTip (50 μg/ml, 2.6 μM) induced 18.0 foci/well, and TPA (1 μg/ml, 1.6 μM) induced 38.0 foci/well. rTipα also induced clonal growth of Bhas 42 cells in cooperation with v-H-ras gene. All the results indicate that homodimer formation by disulfide bonds of cysteine residues is necessary for induction of both TNF-α gene expression and cell transformation with Tipα.

NF-κB activation

The mechanisms of TNF-α gene expression with rTipα can be understood by NF-κB activation, and DNA binding activity of p65 subunit in whole cell extracts was demonstrated in both Bhas 42 and MGT-40 cells (Suganuma *et al*., 2005). rTipα protein induced NF-κB activation about 2-fold over basal levels at a concentration of 100 μg/ml in both cells, while rdel-Tipα did not. As Fig. 5A shows, NF-κB p65 subunit was clearly translocated into nuclei in MGT-40 cells. Furthermore, activation of NF-κB by Tipα protein was associated with down-regulation of IκB 0.4 from the basal level, a down-

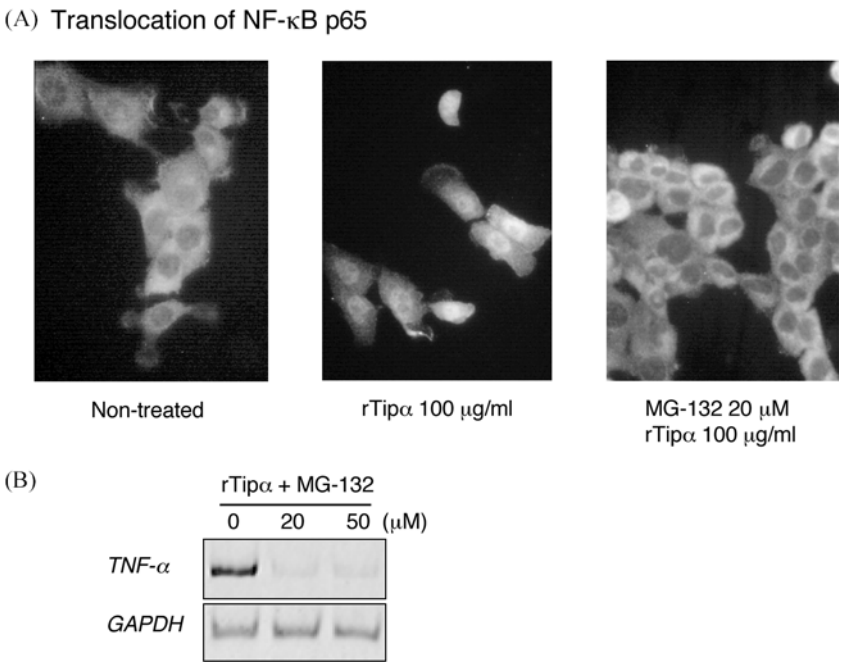

Fig. 5. NF-κB activation by Tipα protein. A. Translocation of NF-κB p65 into nuclei by treatment with rTipα, and pretreament with proteasome inhibitor MG-132 inhibited its translocation. B. MG-132 abrogated TNF-α gene expression induced by rTipα.

regulation that was abrogated by pretreatment with a proteasome inhibitor, MG-132. Pretreatment with MG-132 clearly inhibited both translocation of NF-κB p65 subunit into nuclei and TNF-α gene expression (Fig. 5A and B). The results clearly demonstrated that Tipα protein induced up-regulation of TNF-α in the gastric cells, mediated through NF-κB activation, and then induced cell transformation. Tipα is therefore a new inducer of NF-κB activation. Thus, we think that Tipα protein released from *H. pylori* induces inflammation by NF-κB activation in gastric epithelial cells, and probably induces clonal growth of initiated cells in human stomach.

Our conclusion is supported by various reports that *H. pylori* activates NF-κB in gastric epithelial cells, that activated NF-κB is also found in cells of gastric biopsy that were infected with *H. pylori*, and that activation of NF-κB can stimulate the proliferation of gastric epithelium (Keates *et al*., 1997; Maeda *et al*., 2000). In addition, NF-κB activation is clearly a link between inflammation and cancer: Specific inactivation of IKK/NF-κB pathway can attenuate formation of inflammation-associated tumors in a colitis associated cancer model, and suppressing NF-κB by studies using mouse experimental models - such as colitis-associated cancer - and suppression of NF-κB inhibition with anti-TNF-α treatment resulted in failure to progress to hepatocellular carcinoma in Mdr2-knockout mice (Karin *et al*., 2002; Greten *et al*., 2004; Pikarsky *et al*., 2004). Therefore, we think that NF-κB activation by Tipα plays a key role in stomach carcinogenesis with *H. pylori*.

Weak homology of Tipα to bacterial penicillin binding protein

Since Tipα has no known homologue in other species, the putative functionally important amino acids in Tipα are difficult to predict. In search of a structure-function relationship to Tipα and to predict its ancestral protein, we looked at proteins which have weak homology to Tipα in their primary structures, using Psi-Blast. Numerous Gram-positive bacterial penicillin-binding proteins were found to be weakly homologus to Tipα of *H. pylori* (Kuzuhara *et al*., 2005). Among these, several unique amino acids were conserved and formed a motif-like structure: Three aromatic amino acids, several asparagines and aspartic acids, two hydrophobic amino acids, and one ALV sequence were well conserved between Tipα and the penicillin-binding proteins. It was of interest to note that Tipα is closer to Gram-positive bacterial penicillin-binding proteins than to *H. pylori*, which is a Gram-negative bacterium. This led us to conceive that the ancestor of Tipα is a Gram-positive bacterial penicillin-binding protein, and that at some ancient time the corresponding gene was transferred horizontally from Gram-positive bacteria to *H. pylori*. The amino acids of the motif were mapped in the tertiary structure of penicillin-binding protein PBP2a, which has already been reported (Fig. 6) (Lim and Strynadka, 2002). While the motif is located in the dimerization domain of the penicillin-binding protein PBP2a - and Tipα can also dimerize - the amino acids in the motif are not used directly for dimerization. Therefore, we think that there is a possible target protein common to Tipα

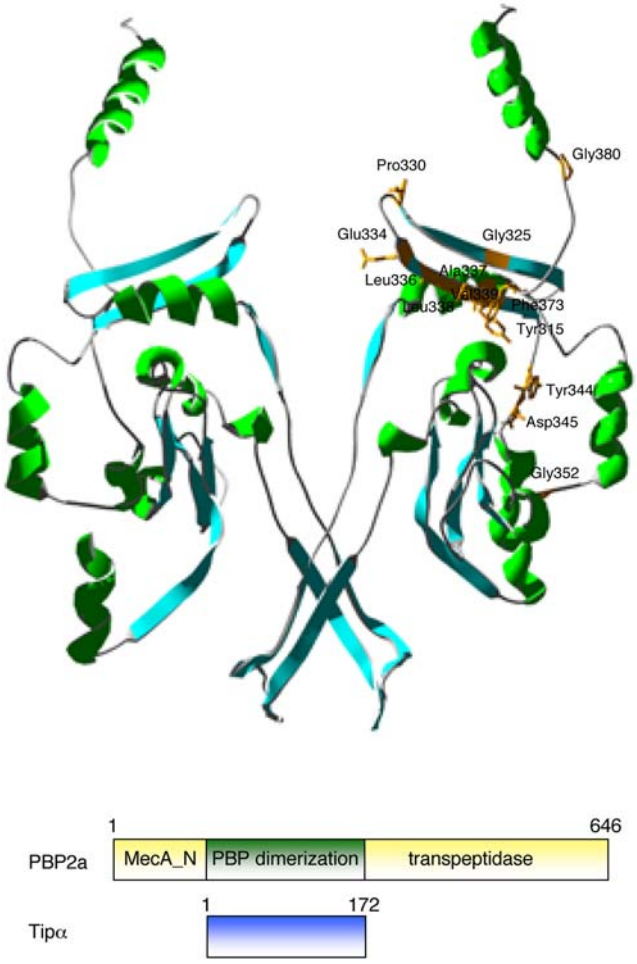

Fig. 6. Mapping of the amino acids conserved between Tipα and penicillin binding proteins on the tertiary structure of PBP2a.

and penicillin-binding proteins and that this target protein interacts with this motif of their dimerization domain.

It is worthwhile to note that many bacterial genes are often horizontally transferred, as can be seen with human oncogenic and drug-resistant genes (Koonin *et al*., 2001). The genes of *cag* PAI and urease accessory protein of *H. pylori* have also been reported to be candidates for horizontal transfer genes (Covacci *et al*., 1999). Although Tipα has not been identified as a horizontally transferred gene by the standard method, we now present Tipα as an additional candidate for horizontal transfer gene.

Conclusion

Tipα is a new NF-κB activating protein of *H. pylori* associated with strong induction of TNF-α in combination with RAS activation (Fig. 7). If *H. pylori* infection occurs in the stomach epithelium in which Ras protein is activated or overexpressed, Tipα dimer is assumed to play a carcinogenic role leading to gastric cancer. However, if *H. pylori* infection occurs in the stomach epithelium without activated Ras protein, Tipα dimer will probably not produce gastric cancer, but only inflammation (gastritis and gastric ulcer). Although activated Ras protein is not often found in human stomach cancer, it is now possible to conceive a new regulatory mechanism of Ras protein with the *let-7* microRNA (Johnson *et al*., 2005). Furthermore, the finding of Tipα has made it possible to classify *H. pylori* into 4 classses: *cag*PAI$^+$ with Tipα$^+$, *cag*PAI$^+$ with Tipα$^-$, *cag* PAI$^-$ with Tipα$^+$, and *cag* PAI$^-$ with Tipα$^-$. These 4 classes provide clues to the differences in carcinogenic potential of *H. pylori*. Based on our evidence that Tipα is a new molecular target for human stomach, further investigation of the mechanism of

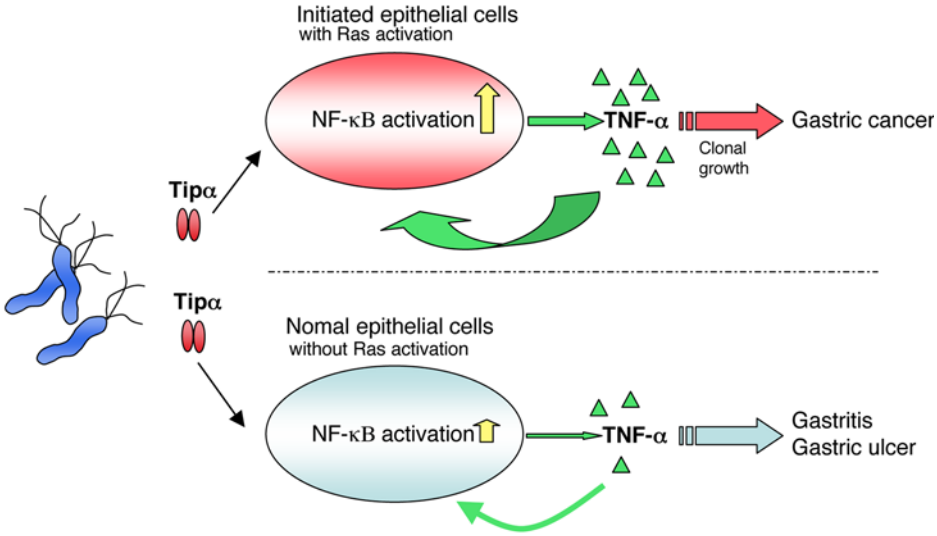

Fig. 7. Schema for carcinogenic role of Tipα in cooperation with Ras protein.

Tipα action will provide a deeper understanding of the process of other inflammation-associated cancer caused by microorganisms.

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