Helicobacter pylori VacA, acting through receptor protein tyrosine phosphatase α, is crucial for CagA phosphorylation in human duodenum carcinoma cell line AZ-521

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ABSTRACT

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INTRODUCTION

Helicobacter pylori is a major causative agent for the development of gastroduodenal diseases, including chronic gastritis, peptic ulcer and gastric cancers (Blaser and Atherton, 2004; Peek and Blaser, 2002). It has been proposed that about 50% of the world’s population is infected with H. pylori, but only a small number of the infected individuals develop severe clinical manifestations such as gastric adenocarcinoma (Wroblewski et al., 2010). Although a number of virulence factors have been found in H. pylori, vacuolating cytotoxin (VacA) and cytotoxin-associated gene A (CagA) are considered to be the major factors in H. pylori-induced diseases (Blaser and Atherton, 2004; Peek and Blaser, 2002).

VacA is a potent cytotoxin secreted by most clinical isolates of H. pylori, and shows pleiotropic actions in cultured gastric epithelial cells, including generation of vacuoles in the cytoplasm, mitochondrial damage leading to apoptosis, and modulation of signal transduction pathways associated with immune responses (Boncristiano et al., 2003; Hisatsune et al., 2007; Isomoto et al., 2010; Nakayama et al., 2004, 2009; Yamasaki et al., 2006). To facilitate their biological actions in host cells, VacA binds to specific surface receptors. We have identified three different cell surface proteins as VacA receptors: receptor protein tyrosine phosphatase α and β (RPTPα and RPTPβ) and low-density lipoprotein receptor-related protein-1 (LRP1) (Yahiro et al., 1999, 2003, 2012). In addition, other molecules, including sphingomyelin, have been reported to serve as VacA receptors (Gupta et al., 2010). Of these VacA receptors, during H. pylori infection, RPTPβ is associated with the development of gastric ulcers in experimental animal models and LRP1 is involved in VacA-dependent autophagy, followed by CagA degradation in infected host cells (Fujikawa et al., 2003; Tsugawa et al., 2012; Yahiro et al., 2012). These data suggest that both receptors are involved in intoxication by VacA. Therefore, we speculate that both receptors, RPTPβ and LRP1, are associated with the development of gastric disorders in H. pylori infection. However, the role of RPTPα in intoxication with VacA is unclear.

Previous studies have shown that RPTPα contributes to activation of cellular Src kinase (Ssrc) and other Src family kinase (Su et al., 1999). It has been shown that Src activity is elevated in RPTPα-overexpressing cultured cells, whereas the opposite was observed in RPTPα-deficient cells (den Hertog et al., 1993; Harder et al., 1998; Su et al., 1999; Zeng et al., 2003; Zheng et al., 1992). Furthermore, it has been reported that Src kinase activity is reduced in RPTPα-
knockout mice (Harder et al., 1998; Ponniah et al., 1999). Therefore, RPTPα is an important physiological regulator of Src. RPTPα can dephosphorylate both phosphorylated tyrosine residues, pTyr530 and pTyr418 (human Src numbering throughout; the inhibitory phosphorylation site and active site of Src, respectively), thereby causing Src activation following autophosphorylation of Tyr418 (Boggon and Eck, 2004; Vacaru and den Hertog, 2010; Zheng et al., 2000). In addition, based on immunohistochemistry using human gastric cancer tissues, it has been suggested that RPTPα is associated with the progression of gastric cancer (Wu et al., 2006).

In the present study, we show the role of RPTPα in VacA intoxication and also demonstrate that VacA is associated with CagA phosphorylation in AZ-521 cells during H. pylori infection. We propose the possibility that VacA induces CagA phosphorylation through RPTPα in AZ-521 cells.

RESULTS
VacA induces Src phosphorylation in vitro
Previous studies have shown that VacA modulates signal transduction pathways in AZ-521 cells (Hisatsune et al., 2007; Nakayama et al., 2009). In this study, we found that VacA enhanced phosphorylation at Tyr418 in Src, but heat-inactivated VacA (iVacA) did not have similar effects (Fig. 1A). We also examined the effect of VacA on Src phosphorylation at Tyr418 using other gastric epithelial cells: AGS cells (a human gastric adenocarcinoma cell line) and NUGC3 cells (a gastric cancer cell line). Although VacA stimulated Src phosphorylation at Tyr418 in NUGC3 cells as well as in AZ-521 cells, we did not find any effects of VacA in AGS cells (Fig. 1B), suggesting that Src phosphorylation induced by VacA is dependent on cell type.

VacA-induced Src phosphorylation is mediated by RPTPα
VacA intoxicates host cells through specific surface receptors. We previously identified three molecules (RPTPα, RPTPβ and LRP1) as VacA receptors (Yahiro et al., 1999, 2003, 2012). To evaluate which of these VacA receptors contributes to Src phosphorylation, we used small-interfering RNA (siRNA)-mediated knockdowns, which targeted each of the VacA receptors, in AZ-521 cells. Although VacA induced phosphorylation at Tyr418 of Src in the presence of control siRNA, VacA did not induce phosphorylation at Tyr418 of Src in siRNA-mediated RPTPα knockdown AZ-521 cells (Fig. 2A). On the other hand, in AZ-521 cells, VacA enhanced phosphorylation at Tyr418 in Src after RPTPβ or LRP1 silencing (Fig. 2B,C). To verify these results, we also examined Src phosphorylation induced by VacA using the RPTPα constitutive-knockdown AZ-521 cells constructed by a shRNA lentiviral expression system (Yahiro et al., 2012). We found that VacA did not enhance phosphorylation at Tyr418 in Src in RPTPα constitutive-knockdown AZ-521 cells (Fig. 3), consistent with the results using the siRNA-mediated RPTPα knockdown AZ-521 cells (Fig. 2A). Taken together, we speculate that RPTPα, but not RPTPβ or LRP1, is involved in VacA-dependent phosphorylation at Tyr418 in Src.

VacA production by H. pylori induces CagA phosphorylation and co-immunoprecipitation with SHP2 phosphatase
Previous studies have shown that CagA is delivered into gastric epithelial cells directly via a type-IV secretion system and then translocated CagA is tyrosine-phosphorylated by Src family kinases, including Src (Odenbreit et al., 2000; Selbach et al., 2006). In this study, we have shown that VacA...
induces phosphorylation at Tyr418 in Src in AZ-521 cells (Figs 1 and 2). We therefore hypothesized that Src phosphorylation induced by VacA is important in CagA phosphorylation during *H. pylori* infection. To evaluate this hypothesis, we examined whether VacA production of *H. pylori* stimulates CagA phosphorylation by challenging cells with wild-type and *vacA* mutant strains in AZ-521 cells. Although total CagA protein, precipitated with anti-CagA antibody, was similar in both wild-type and *vacA* mutant strains, the amount of phosphorylated CagA (pCagA) seen following infection with wild-type *H. pylori* was significantly greater than that seen following infection with a *vacA* mutant strain (Fig. 4A). We next verified the effect of VacA on SHP2 phosphatase, because it has been demonstrated that SHP2 phosphatase specifically binds to pCagA in *H. pylori*-infected host cells (Higashi et al., 2002a). When we carried out immunoprecipitation using anti-CagA antibody following *H. pylori* infection of AZ-521 cells, precipitated SHP2 phosphatase following infection with a *vacA* mutant strain was decreased compared with that seen with wild-type *H. pylori* infection (Fig. 4A). In addition, we also analyzed the effect of VacA on CagA phosphorylation in NUGC3 cells. We found that CagA phosphorylation was stimulated in the early phase (2 h) of infection with wild-type *H. pylori* more than was seen following infection with a *vacA* mutant strain. This was not the case in the late phase of infection (8 h) (Fig. S1).

Several phosphorylation sites in Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs of CagA have been identified. CagA Tyr972 in an EPIYA-C motif is a major Src-phosphorylation site (Backert et al., 2001). A previous study has shown that the EPIYA motif in CagA of wild-type *H. pylori* (strain ATCC43504) is classified as an ABCCC type (Higashi et al., 2002b). To verify the effect of VacA on phosphorylation at Tyr972 in CagA, we used an antibody that recognizes phosphorylated Tyr972 in CagA (Kwok et al., 2007). Infection with wild-type *H. pylori* enhanced phosphorylation of CagA in CagA cells compared with an infection with the *vacA* mutant strain (Fig. 4B), suggesting that VacA might be specifically associated with phosphorylation at Tyr972 and its corresponding sites within EPIYA-C motifs of CagA in *H. pylori* infection. To demonstrate VacA-induced CagA phosphorylation...
VacA (60 nM and 120 nM), but not iVacA, supplementation during infection with a vacA mutant strain significantly increased vacA phosphorylation, compared with that in control siRNA-transfected wild-type AZ-521 cells; KD, constitutive-knockdown AZ-521 cells.

Next, we assessed whether VacA induces CagA phosphorylation by using hemagglutinin (HA)-tagged cagA gene-transfected AZ-521 cells using 120 nM VacA to induce the effects of VacA on CagA phosphorylation (Higashi et al., 2002b). In cells expressing HA-tagged CagA, immunoprecipitation using anti-CagA antibody revealed that CagA phosphorylation was enhanced by VacA, but not iVacA (Fig. 5B), supporting our hypothesis that VacA mediates CagA phosphorylation.

VacA induces CagA phosphorylation in cagA gene-transfected cells in an RPTPα-dependent manner

Next, we examined the effect of RPTPα silencing on VacA-induced CagA phosphorylation in cells infected with wild-type H. pylori. We found that silencing of the RPTPα gene with siRNA resulted in a significant reduction of VacA-induced CagA phosphorylation, compared with that in control siRNA-transfected cells. A prior study showed that β1 integrin is associated with CagA translocation in H. pylori-infected cells through its interaction with CagL, a component of the type IV secretion apparatus of H. pylori, resulting in induction of phosphorylation in both of the amount of pCagA (Fig. 5A), indicating that VacA (60 and 120 nM) induces CagA phosphorylation. In addition, we also investigated the effect of VacA supplementation using NUGC3 cells during infection with a vacA mutant. We found that VacA (120 nM) but not iVacA significantly increased the amount of pCagA in the same manner as seen in AZ-521 cells (Fig. S1). Next, we assayed whether VacA induces CagA phosphorylation by using phospho-Src signal intensity was normalized to total Src. Data were analyzed by two-tailed Student’s t-test. Results are representative of five independent experiments and data are means±s.d. values from triplicate experiments with an n=5 per experiment. *P<0.01 (vs t=0). IB, immunoblotting.
FAK and Src (Kwok et al., 2007). In addition, they speculated that interaction between CagL and the β1-integrin–FAK–Src signaling pathway was associated with CagA phosphorylation (Kwok et al., 2007). When we examined the effect of β1-integrin gene silencing on VacA-induced CagA phosphorylation in cells infected with wild-type H. pylori, we found that silencing of the β1-integrin gene mediated by siRNA did not result in differences of VacA-induced CagA phosphorylation compared to control siRNA-transfected cells (Fig. 6A), suggesting that RPTPα is responsible for CagA phosphorylation induced by VacA in AZ-521 cells. In addition, we assessed whether RPTPα is crucial for CagA phosphorylation by VacA using AZ-521 cells co-transfected with RPTPα siRNA and plasmid harboring an HA-tagged cagA gene. We found that VacA did not induce CagA phosphorylation in AZ-521 cells co-transfected with RPTPα siRNA and plasmid harboring an HA-tagged cagA gene (Fig. 6B). Taken together, these results indicate that RPTPα contributes to the VacA-induced CagA phosphorylation in AZ-521 cells during H. pylori infection.

DISCUSSION

Several VacA receptors have been identified and their roles in the pathogenicity of H. pylori have been characterized (Fujikawa et al., 2003; Isomoto et al., 2010; Tsuchiya et al., 2012; Yahiro et al., 2012). In this study, we provided new insights into the function of RPTPα in VacA-induced Src phosphorylation in AZ-521 cells (Fig. 1A). When we examined the effects of three VacA receptors, i.e. RPTPα, RPTPβ and LRP1, VacA did not induce Src phosphorylation in siRNA-mediated RPTPα knockout AZ-521 cells, indicating that VacA induces the phosphorylation of Tyr418 in Src through RPTPα, rather than through RPTPβ and LRP1. We therefore propose that binding of VacA to the extracellular domain of RPTPα triggers events leading to the phosphorylation of Tyr418 in Src; this phosphorylation induces subsequent actions in host cells, including CagA phosphorylation. Previous studies have indicated that RPTPα, through its Tyr789 residue, interacts with Src, leading to dephosphorylation of Tyr527 in the C-terminal of Src, autophosphorylation of Tyr418 and activation of Src (Bagrodia et al., 1991; den Hertog et al., 1993; Su et al., 1999; Zheng et al., 1992, 2000). The importance of Tyr789 in RPTPα is...
Fig. 6. VacA-induced CagA phosphorylation is mediated through RPTPsiRNA. (A) siRNA-transfected AZ-521 cells were infected with wild-type H. pylori strain at 37°C, 5% CO2 for 9 h at MOI of 100. Translocated CagA proteins were recovered by immunoprecipitation (IP) using anti-CagA antibody. Signals were generated using anti-phosphotyrosine (pY) and anti-CagA antibodies. To quantify the amount of pCagA, signals were normalized to precipitated CagA protein. The effects of siRNA were validated by western blotting using specific antibodies. α-tubulin served as a loading control. The results are mean±s.d. values of five independent experiments. NC, negative control siRNA; RPTPα, RPTPα siRNA; integrin, β1-integrin siRNA. *P<0.01 (vs NC); †P<0.05 (vs NC). (B) AZ-521 cells were co-transfected with siRNA and plasmid harboring HA-tagged cagA gene and cells were incubated at 37°C, 5% CO2 for 24 h. After incubation with 120 nM VacA or heat-inactivated VacA (iVacA), HA-tagged CagA protein was recovered by immunoprecipitation using anti-CagA antibody. Signals were generated using anti-phosphotyrosine (pY) and anti-HA-tag antibodies. To quantify the amount of pCagA, signals were normalized to precipitated CagA protein. The results are mean±s.d. values from triplicate experiments with an n=5 per experiment. *P<0.01 (vs t=0). IB, immunoblotting.

unclear because RPTPsiRNA lacking Tyr789 is still able to dephosphorylate Src Tyr527 (Vacaru and den Hertog, 2010; Yang et al., 2002). Binding of Src to Ser204 of RPTPα is required for phosphorylation of Src Tyr418 (Vacaru and den Hertog, 2010). Thus, multiple steps involving RPTPsiRNA are required for its interaction with Src. RPTPsiRNA, including RPTPsiRNA, are believed to express their specific functions by binding of the extracellular ligand, followed by the modulation of downstream molecules, including Src (Stoker, 2005). Although extracellular binding molecules to RPTPsiRNA have been reported, including VacA (Zeng et al., 1999; Yahiro et al., 2003), interaction mechanisms between those molecules and RPTPsiRNA are not yet available. Further studies are needed to determine how VacA affects the interaction between RPTPsiRNA and Src.

Previous studies have shown that binding of type-IV secretion systems with β1 integrin or phosphatidyserine plays a key role in the translocation of CagA into AGS cells in H. pylori infection. These translocation mechanisms are involved in crucial pathological actions of CagA in infected host cells (Kwok et al., 2007; Murata-Kamiya et al., 2010). Although understanding the molecular mechanisms of CagA phosphorylation in H. pylori infection is crucial in order to explain its pathogenicity, these mechanisms are still debated. In the present study, we have shown that CagA phosphorylation in AZ-521 cells was significantly reduced following infection with a vacA mutant, compared to infection with wild-type H. pylori (Fig. 4A). Furthermore, the amount of immunoprecipitated SHP2 phosphatase that binds to pCagA was reduced following infection with a vacA mutant strain in AZ-521 cells, compared to infection with wild-type H. pylori (Fig. 4A). Previous studies have shown that SHP2 phosphatase can specifically bind to CagA in a tyrosine-dependent manner at the EPIYA-C and -D motifs (Higashi et al., 2002a; Nagase et al., 2015; Naito et al., 2006). Although several tyrosine phosphorylation sites on CagA EPIYA motifs have been identified, Mueller et al. have proposed that only Src induces phosphorylation at CagA Tyr972, located in the EPIYA-C motif (Mueller et al., 2012). Following this observation, we examined the stimulatory effect of VacA on CagA phosphorylation at Tyr972 in AZ-521 cells infected with wild-type and vacA mutant strains. Following infection with wild-type and vacA mutant strains, translocated CagA proteins in AZ-521 cells were similar in amount, but phosphorylated Tyr972 in CagA VacA following infection with a vacA mutant strain was significantly reduced compared to infection with wild-type H. pylori (Fig. 4B). Thus, VacA promotes phosphorylation at Tyr972 in CagA. Therefore, we speculate that reduction of precipitated SHP2 phosphatase following infection with a vacA mutant strain is due to the reduction of CagA phosphorylation at Tyr972 in an EPIYA-C motif in AZ-521 cells. As shown in Fig. 5A, supplementation of VacA significantly induced CagA phosphorylation in the vacA gene-disrupted mutant, compared with control or with supplementation of iVacA. However,
detailed information is currently lacking about the physiological concentration of VacA during \textit{H. pylori} infection or relative concentration at the \textit{H. pylori}-infected host-cell surface. Previous studies have indicated that the concentration of VacA (120 nM) used in this study can induce cell vacuolation and cell death (Yahiro et al., 1997, 2012; Yamasaki et al., 2006). These studies on VacA-dependent cell death are performed in the presence of purified VacA without CagA. In addition, Jain et al. have shown VacA-induced mitochondrial fragmentation, leading to mitochondrial dysfunction and resulting in cell death of AZ-521 cells at 8 h post-infection with \textit{H. pylori} strain (Jain et al., 2011). Thus, these observations indicate that VacA can induce mitochondrial dysfunction, leading to cell death. In this study, we showed that VacA-induced CagA phosphorylation was detected at 2 h post-infection with \textit{H. pylori} infections in NUGC3 cells (Fig. S1). When we also evaluated VacA-induced CagA phosphorylation in AZ-521 cells in more detail, we found that the amount of pCagA at 2 h post-infection with wild-type \textit{H. pylori} was significantly higher than following infection with a vacA mutant (data not shown). Therefore, we speculate that VacA-induced CagA phosphorylation in AZ-521 and NUGC3 cells occurs as an early phase of infection prior to VacA-dependent cell death. The VacA concentrations needed to accelerate CagA phosphorylation and detailed molecular mechanisms on VacA functions in host cells during \textit{H. pylori} infection need to be examined.

Many studies on the pathological mechanisms of \textit{H. pylori} have been conducted using several gastric epithelial cell lines, including AGS cells. It has been shown that AZ-521 cells exhibit different biological properties compared with other cell lines that are used in studies of \textit{H. pylori}. However, we tried to use AZ-521 cells because this cell line could be useful for the identification of currently unknown VacA-induced functions owing to its sensitivity to VacA (Radin et al., 2011, 2014). Whether VacA-induced CagA phosphorylation is a cell-specific event remains to be determined. In this regard, in AGS cells, Asahi et al. have shown that VacA is positively involved in CagA phosphorylation in the early phase of \textit{H. pylori} infection (4 h), but Argent et al. have demonstrated the opposite finding, that VacA does not affect CagA phosphorylation (Argent et al., 2008; Asahi et al., 2003). When we also examined the effect of VacA on CagA phosphorylation in NUGC3 cells, in the early phase of infection (2 h) the amount of pCagA following infection with wild-type \textit{H. pylori} increased by more than that seen following infection with a vacA mutant. However, in the late phase of infection (8 h) there was no difference between the two treatment groups (Fig. S1), indicating that different levels of VacA-induced CagA phosphorylation are observed in different cell lines. In addition, previous studies have shown that VacA has suppressive effects on the actions of CagA in host cells (Ricci et al., 1996; Tegtmeier et al., 2009). In one instance, VacA suppressed CagA-dependent cell elongation in AGS and MKN28 cells by inhibition of Erk1/2 activation (Tegtmeier et al., 2009). However, the action of VacA stimulated the activation of Erk1/2 in other types of cell lines (AZ-521 and TMK1) (Mitsuno et al., 2001; Yahiro et al., 2015). Although the differences in the effects of VacA on CagA responses in each of the cell lines remain unclear, these results indicate that the action of VacA in host cells occurs in a cell-type-specific manner.

In summary, we have shown that VacA promotes CagA phosphorylation through RPTPα in AZ-521 cells (Fig. 7). Our findings demonstrate the possibility that VacA induces CagA phosphorylation in AZ-521 cells during \textit{H. pylori} infection through the VacA-RPTPα-Src signaling pathway. Activation of CagA by a VacA-dependent pathway in AZ-521 cells seems to be important in the pathogenesis seen in \textit{H. pylori} infection.
Bacteria were cultured in Brucella broth (BD, Franklin Lakes, NJ, USA) supplemented with 10% FBS and 10 µg/ml vancomycin at 37°C for 48 h with shaking under microaerobic conditions. After preparation, bacteria were added to AZ-521 cells at a multiplicity of infection (MOI) of 100 and were incubated at 37°C, 5% CO2 for the indicated times. After incubation, AZ-521 cells were washed twice with cold phosphate-buffered saline (PBS) and the whole cell lysate was prepared as previously described (Asahi et al., 2000).

**Immunoprecipitation**

To detect pCagA or pCagA/SHP2 phosphatase complex in the H. pylori-infected cells, we performed immunoprecipitation as previously described (Asahi et al., 2000). In brief, proteins were precipitated using anti-CagA antibody (1:100) at 4°C and precipitated proteins were recovered using nProtein A Sepharose 4 Fast Flow (GE Healthcare, Uppsala, Sweden) according to the manufacturer’s instructions. The precipitates were washed five times with lysis buffer (50 mM Tris-HCl pH 7.4, 1% Triton X-100, 5 mM EDTA and 1 mM Na3VO4) and once with 10 mM HEPES buffer (pH 8.0), and then eluted in SDS sample buffer (2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01 mg/ml bromophenol blue, 62.5 mM Tris-HCl pH 6.8).

A plasmid harboring an HA-tagged cagA gene (Higashi et al., 2002a,b) was transfected in AZ-521 cells using Lipofectamine 3000 reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions, following which the cells were incubated at 37°C, 5% CO2 for 24 h. After transfection, VacA or iVacA was added and cells were incubated at 37°C, 5% CO2 for 1 h and then pCagA was precipitated with anti-CagA antibody in the same manner as above. Eluted proteins were separated on SDS-PAGE and blotted onto PDVF membranes (Millipore, Darmstadt, Germany). Signals were detected using the specific antibodies with ECL Prime Western Blotting Detection Reagent (GE Healthcare). Quantification of each signal was calculated using Multi Gauge V3.0 (GE Healthcare).

**Effects of VacA on the host-cell molecule**

VacA toxin was prepared as previously described (Yahiro et al., 1999). In brief, VacA was precipitated from culture supernatant with ammonium sulfate and purified using a column conjugated with anti-VacA antibodies. Purified VacA eluted in the void volume of a gel-filtration column. Therefore, we believe that purified VacA is in the oligomeric form. Prior to addition of VacA to the monolayer, VacA was treated with 1 M HCl to dissociate its oligomeric structure and induce activation (Yahiro et al., 1999). VacA or iVacA was added to monolayers without supplements and the culture was incubated at 37°C, 5% CO2 for the indicated times. After incubation, cells were washed twice with cold PBS and lysed with SDS sample buffer. Cell lysates were subjected to SDS-PAGE and proteins were detected using specific antibodies.

**Gene silencing by siRNAs**

Knockdown of RPTPα, RPTPβ or β1-integrin in AZ-521 cells was mediated by specific siRNA (Goeß et al., 2005; Müller et al., 2003; Yahiro et al., 2012). Validated LRPI siRNA and negative-control siRNA were obtained, respectively, from Life Technologies and Sigma-Aldrich. The indicated siRNA was introduced into AZ-521 cells using Lipofectamine RNAiMax transfection reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions, following which the cells were incubated at 37°C, 5% CO2 for 24 h. Expression of target proteins was validated by western blotting using specific antibodies.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


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**Supplementary information available online at**


**References**


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