Differences in *Helicobacter pylori* CagA tyrosine phosphorylation motif patterns between western and East Asian strains, and influences on interleukin-8 secretion

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Helicobacter pylori strains from East Asia have an 'East Asian' type of CagA that is more active and predominantly comprises a single type. Strains from other countries have a 'western' type of CagA, which is less active and comprises many different types generated by intragenomic recombination. Co-culture of AGS gastric epithelial cells with isolates of western strains that displayed microevolution in CagA showed that isolates with additional copies of the C motif induced significantly more interleukin (IL)-8 secretion. Co-culture of AGS cells with western and East Asian strains, each expressing CagA with a single copy of the C or D motif, showed that East Asian strains induced significantly more IL-8 secretion. Analysis of the different CagA types from data deposited in GenBank and from the literature showed that western CagA is significantly more likely to undergo duplication of tyrosine phosphorylation motif C than East Asian CagA is of the corresponding D motif. Taken together, the data suggest that the already highly active East Asian CagA with one D motif has no requirement to increase its virulence, whereas the less active western CagA displays flexibility in its capacity to increase its number of tyrosine phosphorylation motifs to become more virulent.

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INTRODUCTION

Helicobacter pylori infects approximately half of the world's population and is the leading cause of gastric and duodenal ulceration and gastric cancer (Atherton, 2006). Around 70 % of strains possess the *cagA* gene, a marker of the *cag* pathogenicity island (PAI), although prevalence is more than 90 % in East Asian countries. The presence of *cagA* has been associated with the development of gastric cancer (Blaser *et al.*, 1995). CagA is transported through the type IV secretion system encoded by the *cag* PAI into the host-cell cytosol, where it becomes phosphorylated by Src and

Abl kinases (Selbach *et al.*, 2002; Stein *et al.*, 2002; Poppe *et al.*, 2007; Tammer *et al.*, 2007). *H. pylori* strains possessing the *cag* PAI induce pro-inflammatory interleukin (IL)-8 secretion from gastric epithelial cells (Tummuru *et al.*, 1995; Censini *et al.*, 1996; Keates *et al.*, 1997); this is thought to be central to the induction of local gastric neutrophil infiltration and so central to disease pathogenesis (Robinson *et al.*, 2007). Recently, *H. pylori* cell-wall peptidoglycan, translocated into the cytosol via the type IV secretion system, has been shown to activate NF- κ B via Nod1 leading to IL-8 secretion (Viala *et al.*, 2004), and CagA, independently of phosphorylation, has also been shown to be involved in IL-8 induction during prolonged incubation (Brandt *et al.*, 2005; Kim *et al.*, 2006).

Phosphorylation of CagA occurs on tyrosine residues, within EPIYA motifs, present within the variable region

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Abbreviations: *cag* PAI, cytotoxin-associated gene pathogenicity island; CI, confidence interval; Csk, C-terminal Src kinase; IL-8, interleukin-8; OR, odds ratio; RAPD-PCR, random amplified polymorphic DNA PCR; TPM, tyrosine phosphorylation motif; VR, *cagA* 3' variable region.

(VR) of the protein. Increasing the number of EPIYA motifs leads to an increase in the level of CagA phosphorylation (Higashi *et al.*, 2002a; Argent *et al.*, 2004, 2008a; Zhang *et al.*, 2005; Naito *et al.*, 2006). The EPIYA motifs have been classified into four types, A, B, C and D (Higashi *et al.*, 2002a), based on the amino acid sequences following each type and all types can be phosphorylated *in vitro*. The A and B motifs can be found in all strains regardless of geographical location. The C motif is additionally present within 'western' strains and the D motif within strains from East Asia (predominantly Korea, China and Japan). The phosphorylated C and D motifs have been shown to interact with SHP-2 phosphatase, leading to cellular rearrangements and the formation of long cellular protrusions in AGS gastric epithelial cells (Higashi *et al.*, 2002b). However, the D motif is more effective at interacting with SHP-2 (Azuma *et al.*, 2004; Higashi *et al.*, 2004; Naito *et al.*, 2006) as it contains the SHP-2 consensus binding motif pY(S/T/A/V/I)X(V/I/L)X(W/F) (De Sousa *et al.*, 2002), whereas the C motif lacks the final part of this motif. The phosphorylated A and B motifs appear to bind to C-terminal Src kinase (Csk), leading to downregulation of Src kinase activity (Tsutsumi *et al.*, 2003; Naito *et al.*, 2006). This may serve to regulate phosphorylation of CagA.

We aimed to determine the effects of *H. pylori* strains with different numbers and types of CagA tyrosine phosphorylation motifs (TPMs), during short and more prolonged incubation times, on IL-8 secretion, and to compare the prevalence of different CagA TPMs between western and East Asian strains.

METHODS

***H. pylori* strains.** *H. pylori* strains were obtained from pinch biopsies during routine endoscopy from patients attending Queen's Medical Centre, Nottingham, UK, with full consent and under ethical approval. Biopsies were spread across blood-agar plates (Oxoid) and 20 single colonies were isolated per strain. Single-colony isolates of *H. pylori* strains were genotyped for the *cagA* VR (Rudi *et al.*, 1998). Isolates that displayed variation in the size of the *cagA* VR were shown to be present within the same strain background by randomly amplified polymorphic DNA (RAPD)-PCR (data not shown) as described previously (Akopyanz *et al.*, 1992). Nucleotide sequencing of *cagA* VRs showed that variation was due to intragenomic recombination of a 102 nt region encoding the C motif. *H. pylori* strains were also obtained from populations in western Scotland (El-Omar *et al.*, 2000a, b; Argent *et al.*, 2008a) and China (Zhang *et al.*, 2005).

AGS gastric epithelial cell co-culture and IL-8 ELISA. AGS cells were seeded into six-well plates at a density of 3×10^5 cells per well and incubated at 37 °C in a 5 % CO₂ air-humidified atmosphere for 2 days before the addition of *H. pylori* strains (m.o.i. 100) and further incubation for 6–48 h. For comparison of *H. pylori* strains expressing western CagA with those expressing East Asian CagA, three wells per six-well plate were inoculated with a western strain and the other three wells were inoculated with an East Asian strain. After incubation, the medium was removed, centrifuged at 15 000 g for 10 min and the amount of IL-8 secreted into the medium was

determined using a DuoSet human IL-8 ELISA kit (R&D Systems; Argent *et al.*, 2004).

TPM patterns in CagA VRs. The sequences of the CagA VR from 980 strains were obtained from GenBank and from publications where no GenBank accession numbers have been provided (Covacci *et al.*, 1993; Tummuru *et al.*, 1993; Evans *et al.*, 1998; Yamaoka *et al.*, 1998; Dong *et al.*, 2002; Stein *et al.*, 2002; Argent *et al.*, 2004, 2005; Zhu *et al.*, 2005; Devi *et al.*, 2006; Choi *et al.*, 2007; Reyes-Leon *et al.*, 2007). The GenBank accession numbers used were: AB003397, AB015404–AB015415, AB017921–AB017923, AB057003–AB057105, AB089318, AB090073–AB090154, AB110957–AB110974, AB116733–AB116768, AB120416–AB120426, AB190932–AB190957, AB246731–AB246743, AB267217–AB267264, AE000511, AE001439, AF043487–AF043490, AF083352, AF202973, AF222807–AF222809, AF247651, AF249275, AF282853, AF289432–AF289464, AF367250, AF367251, AF427098–AF427100, AF479032, AJ832140–AJ832149, AM279288–AM279335, AM292553–AM292599, AM295786–AM295791, AY121840, AY330637, AY330639, AY330642, AY330644, AY550124, AY571191, AY769770–AY769772, CP000241, DQ011619, DQ011620, DQ306710, DQ067454, DQ091000, DQ812087, DQ985738, EF199548, EF202844, EF202845, EF450136–EF450167 and EF444936–EF444938. Strains that displayed microevolution in *cagA* (usually duplication or deletion of TPMs between different isolates of otherwise identical strains) were excluded from the analysis.

Statistics. Analysis of IL-8 secretion data was performed using Student's *t*-test. Analysis of the occurrence of different types of CagA VR was expressed as odds ratios (OR) with a 95 % confidence interval (CI).

RESULTS AND DISCUSSION

Recently, CagA has been shown to play a role in the induction of IL-8 secretion from gastric epithelial cells during prolonged periods of incubation (≥ 36 h). This is independent of CagA phosphorylation, but dependent upon the region possessing the TPMs (Brandt *et al.*, 2005). However, no data exist to show whether increasing the number of TPMs is accompanied by an increase in IL-8 secretion. The phenomenon of microevolution has been used to describe a genetic change in a single gene in one strain from an individual's stomach that potentially leads to a change in phenotype. This has been demonstrated for *cagA* from western strains (Aras *et al.*, 2003; Carroll *et al.*, 2004; Kim *et al.*, 2006; Panayotopoulou *et al.*, 2007; Reyes-Leon *et al.*, 2007; Argent *et al.*, 2008a; and in GenBank accession nos EF450138, EF450139 and EF450145–EF450149). It occurs commonly in these western populations (up to ~20 % of strains) in which there is either intragenomic duplication or deletion of a 102 nt region encoding the C motif, and we have isolated 14 examples of *cagA* microevolution resulting in single or double duplication/deletion of C motifs in *H. pylori* strain collections from the UK (Nottingham and western Scotland; Argent *et al.*, 2008a). We used this phenomenon to investigate whether increased numbers of C motifs, which increase the extent of CagA phosphorylation (Argent *et al.*, 2004, 2008a), led to increased IL-8 secretion from AGS gastric epithelial cells. We co-cultured seven *H. pylori* paired isolates that displayed microevolution within *cagA* with

AGS cells for 6 or 36 h and found that increasing the number of C motifs significantly increased the amount of IL-8 secreted from the cells after prolonged incubation in all cases (Fig. 1). Interestingly, for five of these strains, there was also a significant increase in IL-8 secretion after only 6 h incubation. We have shown previously that the number of EPIYA motifs had no effect on IL-8 secretion after 6 h co-culture (Argent *et al.*, 2004) although these data were obtained using independent strains from South Africa, rather than microevolved isolates, and we also found that IL-8 secretion after 6 h co-culture was unaffected between wild-type strains and their isogenic *cagA* mutants (Argent *et al.*, 2008b). However, Brandt *et al.* (2005) showed that isogenic *cagA* mutants induced significantly less IL-8 secretion than their parental strain after 9 h co-culture, suggesting that CagA may play a role in IL-8 induction, even during short incubation periods.

East Asian *H. pylori* strains possess a D motif within CagA in place of the western C motif and this has been shown to be a potent inducer of SHP-2 phosphatase (Higashi *et al.*, 2002a; Azuma *et al.*, 2004). Co-culture of four western strains from Scotland expressing CagA with ABC motifs alongside four East Asian strains from China expressing CagA with ABD motifs with AGS cells for 6 or 48 h revealed that, in each case, East Asian strains induced significantly more IL-8 secretion (Fig. 2). When the experiment was repeated by incubating each Scottish strain with a different Chinese strain to the initial experiment (see Fig. 2), there was a similar result – the strain with an ABD CagA induced significantly more IL-8 secretion than the strain with an ABC CagA. In total, we assessed *H. pylori* induction of IL-8 secretion from AGS cells from seven Scottish and six Chinese strains and found that after 6 h incubation there was no significant difference ($P=0.35$) between strains with an ABC motif (630 ± 143 pg IL-8

ml⁻¹) and those with an ABD motif (721 ± 201 pg IL-8 ml⁻¹), but after 48 h incubation, a time point known to be important in CagA-induced IL-8 secretion (Brandt *et al.*, 2005), there was a significant ($P=0.015$) increase in IL-8 secretion for the strains with an ABD motif (2512 ± 1166 vs 4617 ± 1389 pg IL-8 ml⁻¹). Although these experiments were conducted using separate strains, rather than using isogenic mutants in which the C and D motifs have been switched, the data suggest that the D motif, as well as being more effective at activating SHP-2, is also capable of inducing more IL-8 secretion than the C motif. CagA has been shown to cause sustained activation of Erk following activation of SHP-2 (Higashi *et al.*, 2004) and CagA-induced activation of IL-8 occurs via the Erk pathway but appears to be independent of SHP-2 (Brandt *et al.*, 2005). It is unclear, therefore, how the D motif may lead to greater IL-8 secretion, but this may be due to the differences in sequence surrounding the EPIYA motifs and in the regions between these motifs. The increase in IL-8 secretion induced by the D motif has important implications *in vivo*, as it suggests that East Asian *H. pylori* strains may induce more inflammation within the stomach and this may lead to an increase in atrophic gastritis and gastric cancer. The incidence of these diseases is significantly higher in Japanese patients infected with East Asian strains compared with Japanese patients infected with western strains (Azuma *et al.*, 2004).

A comparison of the western and East Asian CagA VR types from 980 strains (Table 1) revealed that there were 22 different western types and 14 different East Asian types, with ABC (63.3%) and ABD (88.3%) being the most common in each group, respectively. It has previously been observed that the ABD type of CagA is most common in East Asia, whereas western forms of CagA display more variation (Hatakeyama, 2006), but this has not previously

Fig. 1. Increasing the number of CagA C motifs in isolates of microevolved western strains induces significantly more IL-8 secretion. AGS cells were co-cultured with isolates of *H. pylori* strains from Nottingham (Q102, Q173 and Q241) and from Scotland (AB17, AB29, AB2 and AB12) that displayed variations in the numbers of CagA C motifs but were otherwise identical by RAPD-PCR (data not shown), in triplicate for 6 and 36 h, before the amount of IL-8 secreted into the medium was determined by ELISA. Open bars, CagA ABC (strain Q173, AABC); light-grey bars, CagA ABCC (strain Q173, AABCC); dark-grey bars, CagA ABCCC. *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$ (Student's *t*-test). The experiment was performed in triplicate with similar findings in each case. Results are shown as means $\pm$ SD.

Fig. 2. East Asian *H. pylori* strains induce significantly more IL-8 secretion than western strains. AGS cells were co-cultured with *H. pylori* strains from Scotland (ABC motif; open bars) alongside *H. pylori* strains from China (ABD motif; shaded bars) in triplicate on four six-well plates (four randomly chosen pairs: pair A, Scottish strain AB1 and Chinese strain Z7; pair B, Scottish strain AB3 and Chinese strain Z19; pair C, Scottish strain AB6 and Chinese strain Z23; pair D, Scottish strain AB11 and Chinese strain Z28) for 6 and 48 h before the amount of IL-8 secreted into the medium was determined by ELISA. In all cases, the East Asian ABD strain induced significantly more IL-8 secretion than the western ABC strain after 48 h incubation ($P < 0.01$) and even after 6 h incubation ($P < 0.05$). *, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$ (Student's *t*-test). When the experiment was repeated by changing the pairs (first pair: strains AB1 and Z19; second pair: strains AB3 and Z23; third pair: strains AB6 and Z28; fourth pair: strains AB11 and Z7), the Chinese strain again induced significantly more IL-8 secretion than the Scottish strain. Each experiment was performed in duplicate with similar findings. Results are shown as means $\pm$ SD.

been quantified. Analysis of those strains possessing CagA with two or more C or D motifs revealed that western strains were significantly more likely to have additional C motifs than East Asian strains were to have additional D motifs (OR=50.2, 95% CI 18.3–137.5). There were only four (0.7%) East Asian strains with two D motifs (ABDD, ABDBD and ABDABD types) compared with 109 (26.3%) western strains with two or more C motifs (Table 1). Analysis of those strains possessing CagA with three or more A or B motifs showed that East Asian strains were significantly more likely to have additional copies of these motifs than western strains (OR=2.53, 95% CI 1.37–4.66).

These data clearly show that strains from East Asia predominantly possess the ABD motif: they rarely have additional D motifs and are more likely to gain additional A or B motifs. The D motif has greater affinity to bind SHP-2, cause cytoskeletal alterations (Higashi *et al.*, 2002a;

Table 1. Prevalence of different types of CagA TPM

Western CagA TPMs		East Asian CagA TPMs	
Type	Number	Type	Number
AB*	19	AABD	4
AAB*	1	AAABD	3
AABC	3	AAAD	1
AABCC	1	ABBD	28
AAABC	1	ABD	500
AAC	1	ABDD	2
ABB*	1	ABDABD	1
ABBC	3	ABDBD	1
ABC	262	ABABD	7
ABCC	81	AeBD†	5
ABCCC	19	AD	8
ABCCCC	1	BD	4
ABCCCCC	2	BBD	1
ABCABC	1	BABD	1
ABCB	1		
ABCBC	1		
ABABC	1		
AeBC†	5		
AC	4		
ACC	2		
ACCC	1		
BC	3		

*These types of CagA lack a C or D motif but the sequence following the B motif indicates that these are western CagA, even though some of the strains possessing these motifs were isolated from East Asian patients.

†These types of CagA possess a tandem EPIYA-B motif: E(S/P)IYAEPY(A/T)QVAKKV(N/S)A. One of the five western strains possessed an unassignable motif (EPIYAKVDDL) before the B motif.

Azuma *et al.*, 2004), and may induce greater levels of IL-8 than the corresponding C motif (Fig. 2). It is tempting, therefore, to speculate that there is no requirement for East Asian CagA to increase the number of D motifs, whereas there may be more pressure for western CagA to increase the number of C motifs due to weaker affinity for SHP-2 binding. Increasing the number of C motifs increases the amount of SHP-2 binding to CagA, although an ABCCC type of CagA appears to bind less SHP-2 than an ABD type (Higashi *et al.*, 2002a). Whereas the C and D motifs can bind SHP-2, the A and B motifs have been reported to bind and activate Csk, which then downregulates Src kinase activity to regulate the phosphorylation of CagA (Tsutsumi *et al.*, 2003; Naito *et al.*, 2006). The increase in A and B motifs found in East Asian CagA may serve to regulate the activity of the more active D motif (50% of East Asian strains with two D motifs have additional A and B motifs, compared with only 2.7% of western strains; OR=35.3, 95% CI=3.6–342.4). However, increasing the number of A or B motifs in East Asian CagA had little effect on the binding of Csk, except in two strains that possessed two BD motifs, where there was a significant increase in Csk

binding activity (Naito *et al.*, 2006). It is significant that an ABD motif is present in 88.3% of East Asian strains (OR=4.4, 95% CI 3.2–6.1), supporting the notion that this is the optimal motif for CagA in East Asian strains.

In summary, East Asian CagA predominantly possesses the ABD type and duplication of the D motif is rare, whilst duplication of the A and/or B motif is more common than in western CagA, which frequently duplicates its C motif. This suggests that the highly active ABD form does not need to increase its virulence, whereas the ABC motif, which has much less affinity for SHP-2 and, as we have shown here, is a less potent inducer of IL-8, has a requirement, possibly under certain selective pressures within the stomach, to increase the number of C motifs. The dynamic capacity of western CagA, which may also permit it to decrease the number of C motifs, could allow *H. pylori* to regulate its virulence and thereby induce lower level pro-inflammatory and pro-oncogenic signalling.

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