

described (24). The inner membrane potential was abolished with 25 μ M carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP). Nonimported precursor was removed by digestion with proteinase K (200 μ g per milliliter of import buffer) for 15 nin at 4°C, followed by addition of 1 mM phenylmethylsulfonyl fluoride (PMSF). Proper insertion of radioactive precursors into the inner membrane was verified by checking resistance to extraction by 100 mM Ma₂CO₃ (25) and resistance to proteinase K in mitoplasts (26).

- 14. For cross-linking, in vitro import was for 10 min at 20°C, and nonimported precursor was digested with proteinase K (30 µg/ml). Mitochondria were washed, resuspended at 1 mg/ml in import buffer, and incubated with 1 mM dithiobis(succinimidy)propionate) (DSP) for 30 min at 4°C followed by a quench with 100 mM tris-base, pH 8.0. For immunoprecipitation, solubilized mitochondria were incubated with monospecific polyclonal antibodies coupled to protein A-Sepharose (27).
- 15. For the multicopy suppressor screen, a yeast genomic DNA library in a 2μ , *URA3* vector was transformed into the temperature-sensitive yeast strains tim10-1 and tim12-1 (28). Transformants were grown at 25°C for 24 hours and then shifted to 37°C. Of 5×10^5 transformants in strain tim12-1, 300 transformants grew at 37°C. Subcloning of the genomic fragment carried by the suppressing plasmid S12 identified *TIM22* as the extragenic suppressor for the *tim12-1* mutation. Of 5×10^5 transformants grew at 37°C, all of them contained only *TIM10* as the "suppressor" gene.
- 16. A hexahistidine tag was added to the COOH terminus of Tim10p by PCR amplification of TIM10 with a primer that inserted six histidine codons immediately upstream of the stop codon. The PCR fragment was subcloned into YCplac111 (creating pTIM10H6) and then sequenced. Strain Tim10H6 was constructed by transformation of strain Δ tim10[*TIM10*] with pTIM10H6 and subsequent removal of the plasmid carrying the wild-type TIM10 gene on media containing 5-fluoro-orotic acid. To purify Tim10H6p and associated proteins, we solubilized mitochondrial protein (2 mg/ml) in 0.5% digitonin and 20 mM imidazole and incubated it with Ni2+-nitrilotriacetic acid-Sepharose (Qiagen, Hilden, Germany). Polyclonal antibodies against Tim10p or Tim12p were raised against the recombinant proteins Tim10p that had been cleaved from a glutathione-S-transferase-Tim10p fusion or a thioredoxin-Tim12p fusion protein, respectively. Polyclonal antibodies against Tim22p were raised against a thioredoxin-Tim22p fusion protein in which the first 16 amino acids of Tim22p had been inserted between amino acids 34 and 35 of thioredoxin.
- A. Tzagoloff, J. Yue, J. Jang, M. F. Paul, J. Biol. Chem. 269, 26144 (1994).
- D. M. Engelman and T. A. Steitz, *Cell* **23**, 411 (1981).
 C. Guthrie and G. R. Fink, Eds., *Methods Enzymol.* **194**, 1–863 (1991).
- R. R. Staples and C. L. Dieckmann, *Genetics* 135, 981 (1993).
- 21. D. Muhlrad, R. Hunter, R. Parker, Yeast 8, 79 (1992).
- 22. R. D. Geitz and R. H. Schiestl, ibid. 7, 253 (1991).
- 23. B. S. Glick and L. Pon, *Methods Enzymol.* **260**, 213 (1995).
- 24. C. Wachter, G. Schatz, B. S. Glick, *Mol. Biol. Cell* 5, 465 (1994).
- Y. Fujiki, A. L. Hubbard, S. Fowler, P. B. Lazarow, J. Cell Biol. 93, 97 (1982).
- 26. B. S. Glick et al., Cell 69, 809 (1992).
- 27. S. Rospert, S. Muller, G. Schatz, B. S. Glick, *J. Biol. Chem.* **269**, 17279 (1994).
- 28. S. B. Helliwell et al., Mol. Biol. Cell 5, 105 (1994).
- 29. We thank N. Pfanner for antiserum against yeast AAC and phosphate carrier and for plasmid pGEM4-PIC for in vitro transcription; F. Palmieri for antiserum against yeast dicarboxylate carrier; S. Helliwell and M. Hall for the yeast genomic library; and N. Kralli, S. Merchant, and members of the Schatz and Schweyen laboratories for helpful discussions. This study was supported by grants from the Swiss National Science Foundation (G.S.), the Austrian Fonds

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Helicobacter pylori Adhesin Binding Fucosylated Histo-Blood Group Antigens Revealed by Retagging

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The bacterium *Helicobacter pylori* is the causative agent for peptic ulcer disease. Bacterial adherence to the human gastric epithelial lining is mediated by the fucosylated Lewis b (Le^b) histo-blood group antigen. The Le^b-binding adhesin, BabA, was purified by receptor activity–directed affinity tagging. The bacterial Le^b-binding phenotype was associated with the presence of the *cag* pathogenicity island among clinical isolates of *H. pylori*. A vaccine strategy based on the BabA adhesin might serve as a means to target the virulent type I strains of *H. pylori*.

Helicobacter pylori, a human-specific gastric pathogen, was first isolated in 1982 (1) and has emerged as the causative agent of chronic active gastritis and peptic ulcer disease (2). Most infected individuals show no clinical symptoms, implicating additional factors, such as genetic predisposition and the genotype of the infecting strain, in disease pathogenesis. Chronic infection is associated with the development of gastric adenocarcinoma, one of the most common types of cancer in humans (3), and *H. pylori* was recently defined as a class 1 carcinogen (4).

The bacterium colonizes the human gastric mucosa by adhering to the mucous epithelial cells and the mucus layer lining the gastric epithelium (5). These adherence

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\$To whom correspondence should be addressed. E-mail: thomas.boren@micro.umu.se properties protect the bacteria from the extreme acidity of the gastric lumen and displacement from the stomach by forces such as those generated by peristalsis and gastric emptying. The fucosylated blood group antigens Lewis b (Le^b) and H-1 (Fig. 1A) mediate adherence of *H. pylori* to human gastric epithelial cells in situ (6).

We have now biochemically characterized and identified the H. pylori blood group antigen-binding adhesin, BabA. Various strains of *H*. *pylori* were analyzed for binding to ¹²⁵I-labeled fucosylated blood group antigens (Fig. 1B) (7, 8). Three of the five strains examined bound Le^b and H-1. The receptor specificities of these strains for the soluble blood group antigens correlate with their adherence properties in situ (6). The prevalence of blood group antigen-binding (BAB) activity was also assessed among 95 recent clinical isolates of H. pylori, and 66% (63 isolates) bound the Le^{b} antigen (7). None of the reference strains or the 95 recent isolates bound to the related Le^a, H-2, Le^{X} , or Le^{Y} antigens (Fig. 1, A and B). These results support previous observations of the receptor specificity of H. pylori for the Leb and H-1 blood group antigens (6) and, in addition, demonstrate the high prevalence of BAB activity among clinical isolates.

Isolates of *H. pylori* are thought to differ in virulence and those from individuals with peptic ulcers most often are type I strains that express the vacuolating cytotoxin A (VacA) and the cytotoxin-associated gene A (CagA) protein (9). By definition, type II strains express neither marker. Twenty-one strains of previously defined type (10) and 73

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of the 95 recent isolates were analyzed for cag genotype (11) and binding to the Le^b antigen. The presence of cagA was associated with bacterial binding to the Le^b antigen; 73% (54/74) of CagA+ strains, compared with 5% (1/20) of the CagA⁻ strains, were positive for binding. The cagA gene is located in the 40-kb cag pathogenicity island (PAI), which contains genes that encode proteins with similarities to components of secretion systems (12). However, a deletion of the entire PAI that we engineered into a type I Le^b antigen–binding strain resulted in no reduction in Le^b antigen-binding activity (13). Thus, the epidemiological association between CagA⁺ status and Le^b antigenbinding activity is not mechanistic.

We next determined the affinity constant (K_a) for the BabA-Le^b interaction by performing receptor displacement analyses (Fig. 1C). These results showed that the receptor-adhesin complex was formed under conditions of equilibrium. Most of the cells (>90%) of the bacterial population exhibited BAB activity, as determined with the use of confocal microscopy and fluorescent Le^b antigen (14). The K_a value for formation of the Le^b antigen-BabA complex was $\sim 1 \times 10^{10} \text{ M}^{-1}$ (Fig. 1D) (15). The number of Le^b glycoconjugate molecules bound to BabA was calculated as \sim 500 per bacterial cell, similar to the number of fimbriae organelles on the surface of Escherichia coli (16).

The localization of BabA on the bacterial cell surface was investigated by immunogold electron microscopy. The BabA adhesin was detected on the bacterial cell outer membrane by probing with the Le^b antigen, but not with the Le^a antigen (Fig. 2, A and B) (14). No gold particles were located on the flagellar sheath, suggesting that, despite their continuity, the membranes of the cell surface and sheath differ in protein composition.

The molecular mass of the BabA protein was characterized by receptor overlay analysis. BAB activity corresponded to a single 75-kD band (Fig. 2C) (17); a 40-kD band also detected is probably endogenous peroxidase, possibly the 39-kD HP1461 peroxidase (18), because it stained without the Le^b conjugate overlay. The panel of strains from Sweden, Australia, and South America showed a conserved molecular mass of BabA (Fig. 2D).

Because the BabA protein is not abundant (Fig. 1D), we developed a combined ligand identification and purification technique, termed receptor activity-directed affinity tagging (ReTagging). Cross-linking agents with radiolabeled donative tags have previously been used for characterization of receptor ligands (19). However, for ReTagging, the Le^b glycoconjugate was equipped with an affinity tag-donating cross-linker structure. The modified Le^b glycoconjugate directed the targeted transfer of the affinity tag (biotin) to the BabA protein by virtue of its receptor activity (Fig. 3, A and B). After cross-linking, the covalently attached biotin tag was used to identify the adhesin with streptavidin (Fig. 2E) (20). One biotin-tagged protein of 75 kD was detected in several strains, consistent with the results of the overlay analysis (Fig. 2, C and D). More generally, ReTagging should

Fig. 1. Biochemical characterization of the blood group antigenbinding (BAB) activity of H. pylori. (A) The fucosylated blood group antigens. The H antigen (defining group O in the ABO blood group system) presents the Fuca1.2 residue (no. 1) in the core chain, the Lea antigen instead presents the Fuca1.4 residue (no. 2), and the difucosylated Le^b antigen presents both fucose residues. The equivalent H-2, Lex, and Le^Y antigens differ prove useful for diverse studies of interactions whether in infectious disease, inflammation processes, or cell differentiation and development.

The high specificity of the ReTagging technique provided a means for affinity-purification of the adhesin protein. After cross-linking, bacteria were solubilized in SDS sample buffer, streptavidin-coated magnetic beads were added to the solubilized proteins, and biotin-tagged BabA protein was extracted (Fig. 3C) (21). The sequences of the 20 NH₂-terminal amino acids of the BabA ad-



respectively by having a central β 1.4 linkage, compared with the β 1.3 linkage in the previous series (30). The fucosylated blood group antigens are typically found on red blood cells where they define the ABO blood group system, but they are also expressed on the epithelial cell surfaces as histo-blood group antigens (30). Gal, galactose; GlcNAc, *N*-acetylglucosamine; Glc, glucose. (**B**) Bacterial binding to soluble blood group antigens. Five *H. pylori* strains (7) were incubated with ¹²⁵I-labeled blood group antigen glycoconjugates (8). Solid bars, Le^b; open bars, H-1; striped (third) bars, Le^a; hatched (fourth) bars, H-2 plus Le^Y (**C**) Receptor displacement assay. Strain CCUG17875 was incubated for 1 hour with 10 ng of ¹²⁵I-labeled Le^b conjugate, and the resulting complex was then challenged with an excess of unlabeled Le^b or Le^a conjugate. The remaining radioactivity in the bacterial pellet was measured (8). The Le^b conjugate patter anged from 50 ng to 8 μ g. (**D**) Scatchard analysis of the *H. pylori*-Le^b antigen interaction. Binding of strain CCUG 17875 to the Le^b antigen (8) was measured at Le^b conjugate concentrations of 10 to 260 ng/ml, yielding a K_a value of 8 × 10⁹ M⁻¹ (15).

Fig. 2. Localization and characterization of the BabA adhesin. (A and B) Electron microscopy of cells of H. pylori strain CCUG17875 exposed to biotinylated Leb or Lea glycoconjugates, respectively. After washing, bacteria were incubated with 10-nm gold-labeled antibodies to biotin (ICN, Costa Mesa, California), counterstained, and air-dried onto formvar-coated copper grids (14). Bar, 1 µm. (C) Characterization of the molecular mass of the BabA adhesin by receptor overlay analysis (17). SDS-solubilized protein extracts of strain CCUG17875 were separated by SDS-PAGE and transferred to a PVDF membrane, which was then incubated with biotinylated Leb glycoconjugate (lane 1) or biotinylated albumin (lane 2), followed by peroxidase-streptavidin. The positions of molecular size markers (in kilodaltons) are indicated on the left. (D) Receptor overlay analysis of BabA adhesins from various strains. Lanes 1 to 4: A5 (Sweden), P466 (South America), CCUG17875 (Australia), and MO19



(United States), respectively. The lack of a Le^b antigen–binding band with MO19 is consistent with this strain's lack of BAB activity (Fig. 1B). (E) Receptor activity–directed affinity tagging (ReTagging) (20) of BabA from various strains. Lanes are as in (D). Results are consistent with those in (D).

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hesins from an Australian and a Swedish strain were found to be identical, and were used to construct degenerate polymerase chain reaction (PCR) primers for cloning purposes (22). Two sets of clones were iden-

Fig. 3. ReTagging and purification of the BabA adhesin. (A) A multifunctional cross-linking agent was conjugated to the Le^b alvcoconiugate. The Sulfo-SBED cross-linker contains an NHS group for conjugation to the protein core of the glycoconjugate, a central disulfide bond, a photoreactive aryl azide group, and a biotin side group (star) (20). (B) The crosslinker-labeled Leb glycoconjugate is incubated with H. pylori so that binding to the bacteria brings the cross-linker molecule close to the



tified that encode two proteins with almost

identical NH2-terminal domains and com-

pletely identical COOH-terminal domains

 $(\sim 300 \text{ amino acids})$, but with divergent cen-

tral regions (Fig. 4A). For the identification

adhesin protein. When the cells are subjected to ultraviolet (UV) irradiation, the photoreactive group forms a covalent bond to structures in the immediate vicinity (that is, the adhesin protein). Exposure to reducing conditions (DTT) results in cleavage of the disulfide bond in the cross-linker. The Le^b glycoconjugate is subsequently released and washed away. (**C**) Consecutive steps in the purification of BabA protein. Lane 1, SDS-PAGE and Coomassie blue staining of untreated bacterial protein extract (control). Lanes 2 and 3, protein extract after the ultraviolet-activated cross-linking reaction; lane 2 shows the Coomassie blue–stained gel and lane 3 shows blot detection of biotin-tagged proteins with peroxidasestreptavidin [the 75-kD BabA protein (asterisk) and some remaining Le^b glycoconjugate of >100 kD are apparent]. Lane 4, blot analysis of the protein extract after treatment with streptavidin-coated magnetic beads; no detectable biotin-tagged adhesin protein remained in the protein extract. Lanes 5 and 6, Coomassie blue–stained gel and blot analysis, respectively, of BabA protein eluted from the streptavidin-coated magnetic beads. Lanes 7 and 8, Coomassie blue–stained gel and blot analysis, respectively, of the protein preparation after final fractionation by preparative SDS-PAGE; BabA is now the dominant protein band (*21*). This band was excised for NH₂-terminal and COOH-terminal sequencing (*21, 23*).



Fig. 4. (A) The translated *babA* and *babB* sequences showing regions of amino acid sequence similarity and heterogeneity in black and white, respectively. The BabA2 signal peptide starts at position –20. Position – 18 of BabB indicates the predicted translational start position. Positions 721 for BabA (78 kD) and 689 for BabB (75 kD) indicate the ends of the open reading frames. **(B)** Nucleotide sequence of the upstream and signal peptide regions of the functional adhesin gene *babA2*. The putative Shine-Dalgarno sequence is underlined. The signal peptide sequences predicted by *babA1* and *babA2* are also shown. The 10-bp insert with a repeat motif (RM) is absent from *babA1*, which is otherwise identical to *babA2*, resulting in elimination of the start codon. A UAA termination codon is present at codon position –24 of the signal peptide region in *babA1*. The accession numbers for *babA1* and *babA2* are AF001388 and AF033654, respectively, with both predicted proteins being 91% identical to HP1243 (*18*). The accession number for *babB* is AF001389, with the predicted protein being 95% identical to HP896 (*18*). Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

of the functional *babA* gene, the BabA adhesin was subjected to large-scale purification by ReTagging, which provided sufficient protein for determination of the sequence of the 41 NH_2 -terminal residues. The DNA sequence of one set of clones encoded this 41-amino acid sequence, and the corresponding gene was designated *babA*. The gene corresponding to the second set of clones was designated *babB*.

The genes corresponding to both sets of clones appear to encode proteins with an 18to 20-residue signal peptide sequence that would be cleaved during secretion to produce the determined NH2-terminal EDD sequences. The calculated molecular size of the mature babA product is 78 kD (23). However, the cloned babA gene lacked an initiation codon at the start of the open reading frame (Fig. 4B). To localize additional babA gene alleles, we screened an ordered cosmid library, and two babA genes and one babB gene were mapped (24). Gene inactivation experiments identified the functional babA gene in strain CCUG17875, which expresses the BabA adhesin; inactivation of the second babA gene, now denoted babA2, resulted in a loss of Le^b antigen-binding activity, whereas inactivation of the original babA gene (babA1) did not affect Leb antigenbinding activity (24). The functional babA2 gene was subsequently amplified by PCR and sequenced (without cloning). The coding region was found to be identical to the previously cloned and sequenced babA1 with the exception of an insert of 10 base pairs (bp) with a repeat motif in the signal peptide sequence, which resulted in the creation of a translational initiation codon (Fig. 4). Sequence analysis of the babA2 gene obtained by PCR amplification and cloning in a plasmid in E. coli revealed frequent deletion of the repeat motif and convergence to the silent babA1 gene, suggesting the presence of hot spots for phenotypic (phase) variation within the bab gene family (25).

The babA and babB coding sequences are highly similar to open reading frames in the sequenced genome of strain 26695 (open reading frames HP1243 and HP896, respectively) (18). The genomic location of babA1 corresponds to that of open reading frame HP1243, as revealed by an almost perfect match between the upstream open reading frame HP1244 (ribosomal protein S18) and the sequence upstream of babA1 from strain CCUG17875. No equivalent genomic location for the babA2 upstream sequence was detected. Absence of the babA2 allele might explain the lack of Le^b antigen-binding activity in strain 26695 (Fig. 1B). Although the cag PAI locus has a _ lower G+C content than the average of 39% for H. pylori (18), babA has a G+C content of 46%. Differences in G+C content may indicate the acquisition of genes and DNA loci from different sources.

The *bab* genes belong to a family of ~ 30 genes whose products show extensive amino acid sequence homology in the NH₂terminal and COOH-terminal domains (18) (Fig. 4A), suggesting possibilities for recombination and consequent changes in the positions of individual genes. Evidence supporting this possibility is provided by the map positions of genes in several strains. In strain 26695, babB is located 5.3 kb from the vacA gene (18). Pulsed-field gel mapping also placed babB near vacA in strain NCTC11637 (26). In contrast, in strain NCTC11638 (24), babA2 is located close to vacA. Recombination between duplicate segments would allow adhesin synthesis to be readily switched on or off. Such a mechanism might be important in determining host specificity during colonization and in bacterial persistence during chronic infection (27).

We propose that BabA-mediated adherence of H. pylori to gastric epithelium plays a critical role in efficient delivery of bacterial virulence factors that damage host tissue either directly or through inflammatory or autoimmune reactions, eventually leading to ulcer disease. Immunization experiments with adhesins of uropathogenic E. coli have demonstrated the potential for the generation of antibodies that inhibit adhesion (28). A vaccine strategy based on the BabA adhesin might possibly target the virulent type I strains of H. pylori, sparing the less virulent strains that may be constituents of the commensal flora (29).

REFERENCES AND NOTES

- 1. J. R. Warren, *Lancet* i, 1273 (1983); B. Marshall, *ibid.*, p. 1273.
- 2. A. Dubois, Emerg. Infect. Dis. 1, 79 (1995).
- 3. M. J. Blaser, Sci. Am. 274, 92 (February 1996).
- 4. R. P. Logan, Lancet 344, 1078 (1994).
- M. J. Blaser, *Trends Microbiol.* 1, 255 (1993); D. E. Kirschner and M. J. Blaser, *J. Theor. Biol.* 176, 281 (1995).
- T. Borén, P. Falk, K. A. Roth, G. Larson, S. Normark, Science 262, 1892 (1993); P. Falk et al., Proc. Natl. Acad. Sci. U.S.A. 90, 2035 (1993); P. Falk, T. Borén, S. Normark, Methods Enzymol. 236, 353 (1994).
- 7. Strain CCUG17875 (Australia) was obtained from CCUG (Göteborg, Sweden). Strain A5, a gastric ulcer isolate, was from Astra Arcus (Södertälje, Sweden). Strains P466 (Peru), MO19 (United States), and NCTC11637 and -11638 were described previously (6). The genome of strain 26695 was recently sequenced (18). The panel of 95 *H. pylori* clinical isolates was obtained from the University Hospital in Uppsala, Sweden. Bacteria were grown at 37°C under 10% CO₂ and 5% O₂ (6) for 2 days to obtain optimal Le^b antigen–binding activity.
- All blood group antigen glycoconjugates used were semisynthetic glycoproteins prepared by the conjugation of purified fucosylated oligosaccharides to human serum albumin (6) [P. D. Rye, *Nature Biotechnol.* **14**, 155 (1996)], and were from IsoSep AB (Tullinge, Sweden). The binding assay (Fig. 1B) was performed as previously described [P. Falk, T. Borén, D.

Haslam, M. G. Caparon, *Methods Cell Biol.* **45**, 161 (1994)] with minor modifications. The H-1, Le⁹, Le⁹, H-2, Le⁸, and Le⁹ glycoconjugates were labeled with ¹²⁵] by the chloramine T method. One milliliter of bacteria (optical density at 600 nm, 0.10) was incubated with 400 ng of ¹²⁵-labeled conjugate (that is, an excess of receptor substrate) for 30 min in phosphate-buffered saline containing 0.5% alburnin and 0.05% Tween-20. After centrifugation, the radioactivity bound to the bacterial pellet was measured with a gamma counter. Binding experiments were reproducible and performed in triplicate. In addition, the extent of BAB activity of each strain was stable.

- A. Covacci et al., Proc. Natl. Acad. Sci. U.S.A. 90, 5791 (1993); M. Marchetti et al., Science 267, 1655 (1995).
- 10. Z. Xiang et al., Infect. Immun. 63, 94 (1995).
- Strains were analyzed for the presence of *cagA* as described [M. K. Tummuru, T. L. Cover, M. J. Blaser, *ibid.* 61, 1799 (1993)].
- S. Censini *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 93, 14648 (1996).
- 13. For the total cag PAI deletion, polymerase chain reaction (PCR) products from both ends of the cag PAI [generated with primers 2F and 4R (0.4 kb), and 24F and 25R (0.6 kb)] were cloned in pBluescript SK (Stratagene, La Jolla, CA). The camR gene [Y. Wang and D. E. Taylor, Gene 94, 23 (1990)] was ligated between the fragments, and the product was used to transform H. pylori strain P119 (31). The cag PAI deletion status of the transformants was verified by their failure either to hybridize with any of several cag region probes or to yield PCR products with primers specific for internal regions of the cag PAI. The sequences of primers 2F and 4R, derived from cosmid 36 (24), and of 24F and 25R (9) are as follows: 2F, 5'-ACATTTTGGCTAAATAAACGCTG; 4R, 5'-TCTCCATGTTGCCATTATGCT; 24F, 5' GGAATTATCACACCTTATAATGCCC; and 25R, 5'-TCATGCGAGCGGCGATGTG
- Confocal microscopy was performed with a Nikon/ Multiprobe 2001 instrument (Molecular Dynamics, Sunnyvale, CA). Electron microscopy was performed with a JEOL 100 CX instrument.
- 15. A. G. Scatchard, Ann. N.Y. Acad. Sci. **51**, 600 (1949).
- O. Mol and B. Oudega, FEMS Microbiol. Rev. 19, 25 (1996).
- 17. Cell extracts were solubilized in SDS sample buffer (without mercaptoethanol) at 37°C for 10 min and then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated overnight with biotinylated Le^b glycoconjugate (1 μg/ml) or biotinylated albumin (negative control), prepared as previously described (6). After washing, the biotinylated structures bound by the BabA protein band were visualized with horseradish peroxidasestreptavidin and ECL reagents (Amersham).
- 18. J.-F. Tomb et al., Nature 388, 539 (1997).
- J. Brunner, *Trends Cell Biol.* 6, 154 (1996); J. D. Bleil and P. M. Wassarman, *Proc. Natl. Acad. Sci. U.S.A.* 87, 5563 (1990).
- 20. Bacteria were incubated with Le^b glycoconjugate to which the Sulfo-SBED cross-linker (Pierce, Rock-ville, IL) had been attached by *N*-hydroxysuccinimide ester (NHS). The photoreactive aryl azide cross-linker group was activated by ultraviolet irradiation. Bacteria were then washed with phosphate-buffered saline (pH 7.6) containing 0.05% Tween-20, protease inhibitors (EDTA and benzamidine), and 50 mM dithiothreitol (DTT). Bacterial proteins were separated by SDS-PAGE and transferred to a PVDF membrane, after which the biotin-tagged BabA protein was detected with peroxidase-streptavidin and ECL reagents (Fig. 2E).
- 21. Strains CCUG17875 and A5 were first processed by cross-linking and DTT treatment as described (20), and then solubilized in SDS sample buffer. The biotin-tagged BabA protein was then extracted with strepta-vidin-coated magnetic beads (Advanced Magnetics, Cambridge, MA). The beads were boiled in SDS sample buffer, and eluted proteins were alkylated. The protein preparation was further fractionated by preparative

SDS-PAGE (Prep-Cell 491; Bio-Rad, Hercules, CA). The pooled BabA preparation was finally separated by SDS-PAGE and transferred to a PVDF membrane. The BabA band (15 pmol) was excised and the protein was subjected to NH2-terminal sequencing (41 amino acids) with a Procise 494 instrument (Applied Biosystems Foster City, CA). The biotin-tagged BabA adhesin was purified >3000-fold from the cell extract, and the yield was ~20%. The results of the Scatchard analysis (Fig. 1D) indicate that the level of BabA would be about five times higher compared to the resulting biotin-tagged BabA adhesin. The Retagging efficiency is dependent on the combined affinity and specificity in the receptorligand interaction and, in addition, the steric availability of the cross-linker structure. However, in our hands, a nonsaturated efficiency results in low background of unspecific biotin-tagging (Fig. 2E).

- 22. The NH₂-terminal sequence of BabA was used to design degenerate oligonucleotides for PCR amplification of a 59–bp fragment from the CCUG17875 chromosome. This 59-bp fragment was used to probe a low-copy number plasmid (pACYC184) library of CCUG17875 chromosomal DNA partially digested with Sau 3A.
- 23. The integrity and identity of the COOH-terminal domain was verified by COOH-terminal peptide sequencing. The BabA band (85 pmol) (21) was excised, and the last five amino acid residues were determined to be YV(F)A-, compared with YVFAY predicted from the gene sequence, with a Procise 494C instrument (Applied Biosystems). [(F), weak signal; -, amino acid not identified, probably because of derivatization during analysis]
 - An ordered cosmid library of strain NCTC11638 [N. O. Bukanov and D. E. Berg, Mol. Microbiol. 11, 509 (1994)], in which vacA is located on cosmids and 2, was probed with oligonucleotides for specific segments of the babA (A19) and babB (A18) genes. Whereas babA was mapped to the overlapping cosmids 43 and 44 (babA1) and to cosmids 1 and 2 (babA2), babB was mapped to cosmids 21 and 22. Thus, the bab gene locations perfectly match the RS1 (repetitive sequence 1) positions of the ordered cosmid library. Sequence analysis of strain CCUG 17875 revealed that babA1 and babA2 are identical in the coding region, with the exception of the 10-bp insert (Fig. 4). The sequences of primers A19 and A18 are 5'-GAAGAGGTGCTTTCTTGACCATTAG-CGTTACCCCCGCATTGCGT and 5'-CCATTTGC GCTCTCGTTACTGCCAGGACCACAAGCAGTA-AA, respectively. For generation of the babA deletions, babA, including the babA2 upstream sequence, was amplified with F2 (forward) and R39 (reverse) primers and cloned in pBluescript SK. The vector was linearized with primers R41 and F38. The camR gene (13) was ligated between the fragments, and strain CCUG17875 was transformed and subjected to selection for CamR. The transformants were analyzed for binding to ¹²⁵I-labeled Le^b glycoconjugate, and the location of the camR gene was analyzed by PCR with the upstream primers F2 (babA2) or F44 (babA1) in combination with primer R11. The sequences of the primers are as follows: F2, 5'-CTTAAATATCTCCCTATCCC; R39, 5'-CAAATACACGCTATAGAGCC; R41, 5'-GCGAGC-CTAAAGTTAATGA; F38, 5'-ACGTGGCGAACTTC-CAATTC; F44, 5'-CAGTCAAGCCCAAAGCTATGC; and R11, 5'-CGATTTGATAGCCTACGCTTGTG.
- 25. T. Borén et al., in preparation.
- 26. Pulsed-field gel mapping was performed as described [O. Jiang, K. Hiratsuka, D. E. Taylor, *Mol. Microbiol.* 20, 833 (1996)]. The locations of the *babA* and *babB* genes were analyzed with PCR-generated probes. PCR was performed with the forward primers F43 (*babA*) or F15 (*babB*) in combination with primers R29 and R28, respectively. The sequences of the primers are as follows: F43, 5'-CTTGAG-CAAACTTTTGACCCGAT; F15, 5'-TGGGCCTATA-TCCACTGCAA; R29, 5'-AGTGCCAGCTGTTGA-TTTGTTGCATGTGG; and R28, 5'-TACGCT-CACCCCTTGCTCATAACCA.
- P. Hagblom, E. Segal, E. Billyard, M. So, *Nature* **315**, 156 (1985); R. Haas and T. F. Meyer, *Cell* **44**, 107 (1986)
- 28. S. Langermann et al., Science 276, 607 (1997); dis-

cussed in T. Borén and P. Falk, Sci. Am. Sci. Med. 1, 28 (April 1994); L. S. Tompkins and S. Falkow, Sci ence 267, 1621 (1995).

- 29. M. J. Blaser, Lancet 349, 1020 (1997).
- 30. H. Clausen and S. Hakomi, Vox Sang., 56, 1 (1989). 31. We thank Q. Jiang and D. E. Taylor for analysis of the locations of the babA and babB genes; R. Gilman for
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Cancer Treatment by Targeted Drug Delivery to **Tumor Vasculature in a Mouse Model**

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In vivo selection of phage display libraries was used to isolate peptides that home specifically to tumor blood vessels. When coupled to the anticancer drug doxorubicin, two of these peptides—one containing an α_v integrin-binding Arg-Gly-Asp motif and the other an Asn-Gly-Arg motif-enhanced the efficacy of the drug against human breast cancer xenografts in nude mice and also reduced its toxicity. These results indicate that it may be possible to develop targeted chemotherapy strategies that are based on selective expression of receptors in tumor vasculature.

Endothelial cells in the angiogenic vessels within solid tumors express several proteins that are absent or barely detectable in established blood vessels (1), including α_{i} integrins (2) and receptors for certain angiogenic growth factors (3). We have applied in vivo selection of phage peptide libraries to identify peptides that home selectively to the vasculature of specific organs (4, 5). The results of our studies imply that many tissues have vascular "addresses." To determine whether in vivo selection could be used to target tumor blood vessels. we injected phage peptide libraries into the circulation of nude mice bearing human breast carcinoma xenografts.

Recovery of phage from the tumors led to the identification of three main peptide motifs that targeted the phage into the tumors (6). One motif contained the sequence Arg-Gly-Asp (RGD) (7, 8), embedded in a peptide structure that we have shown to bind selectively to $\alpha_{\nu}\beta_{3}$ and $\alpha_{\nu}\beta_{5}$ integrins (9). Phage carrying this motif, CDCRGDCFC (termed RGD-4C), homes to several tumor types (including carcinoma, sarcoma, and melanoma) in a highly selective manner, and homing is specifically inhibited by the cognate peptide (10).

A second peptide motif that accumulat-

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ed in tumors was derived from a library with the general structure CX₃CX₃CX₃C (X = variable residue, C = cysteine) (6). This peptide, CNGRCVSGCAGRC, contained the sequence Asn-Gly-Arg (NGR), which has been identified as a cell adhesion motif (11). We tested two other peptides that contain the NGR motif but are otherwise differ-

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Fig. 1. Recovery of phage displaying tumor-homing peptides from breast carcinoma xenografts. Phage [10⁹ transducing units (TU)] was injected into the tail vein of mice bearing size-matched MDA-MB-435-derived tumors (~1 cm³) and recovered after perfusion. Mean values for phage recovered from the tumor or control tissue (brain) and the SEM from triplicate platings are shown. (A) Recovery of CNGRCVSGCAGRC phage from

tumor (solid bars) and brain (striped bars), and inhibition of the tumor homing by the soluble peptide CNGRC. (B) Recovery of CGSLVRC phage and inhibition of tumor homing by the soluble peptide CGSLVRC. (C) Recovery of RGD-4C phage (positive control) and unselected phage library mix (negative control). (D) Increasing amounts of the CNGRC soluble peptide were injected with the RGD-4C phage. (E) Increasing amounts of the RGD-4C soluble peptide were injected with the NGR phage. Inhibition of the CNGRCVSGCAGRC phage homing by the CNGRC peptide is shown in (A); inhibition of the RGD-4C phage by the RGD-4C peptide has been reported (10). ent from CNGRCVSGCAGRC: a linear peptide, NGRAHA (11), and a cyclic peptide, CVLNGRMEC. Tumor homing for all three peptides was independent of the tumor type and species; the phage homed to a human breast carcinoma (Fig. 1A), a human Kaposi's sarcoma, and a mouse melanoma (12). We synthesized the minimal cyclic NGR peptide from the CNGRCVSG-CAGRC phage and found that this peptide (CNGRC), when coinjected with the phage, inhibited the accumulation of the CNGR-CVSGCAGRC phage (Fig. 1A) and of the two other NGR-displaying phages in breast carcinoma xenografts (12).

The third motif—Gly-Ser-Leu (GSL) and its permutations-was frequently recovered from screenings using breast carcinoma (6), Kaposi's sarcoma, and malignant melanoma, and homing of the phage was inhibited by the cognate peptide (Fig. 1B). This motif was not studied further here.

The RGD-4C phage homes selectively to breast cancer xenografts (Fig. 1C). This homing can be inhibited by the free RGD-4C peptide (10), but not by the CNGRC peptide, even when this peptide was used in amounts 10 times those that inhibited the homing of the NGR phage (Fig. 1D). Tumor homing of the NGR phage was also partially inhibited by the RGD-4C peptide (Fig. 1E), but this peptide was only 10 to 20% as potent as CNGRC. An unrelated cyclic peptide, GACVFSIAHECGA, had no effect on the tumor-homing ability of either phage (12). Thus, our in vivo screenings yielded two peptide motifs, RGD-4C and NGR, both of which had previously been reported



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