Role of Bacterial Intimin in Colonic Hyperplasia and Inflammation

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Enteropathogenic *Escherichia coli* (EPEC) cells adhere to gut epithelial cells through intimin α: the ligand for a bacterially derived epithelial transmembrane protein called the translocated intimin receptor. *Citrobacter rodentium* colonizes the mouse colon in a similar fashion and uses a different intimin: intimin β. Intimin α was found to costimulate submitogenic signals through the T cell receptor. Dead intimin β^+ *C. rodentium*, intimin α–transfected *C. rodentium* or *E. coli* strain K12, and EPEC induced mucosal hyperplasia identical to that caused by *C. rodentium* live infection, as well as a massive T helper cell–type 1 immune response in the colonic mucosa. Mutation of cysteine-937 of intimin to alanine reduced costimulatory activity in vitro and prevented immunopathology in vivo. The mucosal changes elicited by *C. rodentium* were interferon-γ–dependent. Immunopathology induced by intimin enables the bacteria to promote conditions that are favorable for increased microbial colonization.

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of infantile diarrhea in humans. The mouse homolog of EPEC, *Citrobacter rodentium*, causes colonic hyperplasia in young mice. EPEC and *C. rodentium* colonize the surface of epithelial cells in the intestine. They produce an attaching and effacing (AE) lesion on the epithelial cell lumenal surface, using a type III secretion system to secrete several proteins encoded by the chromosomal locus of enterocyte effacement (*1*). These proteins include EspA, EspB, and EspD and produce changes in host cell cytoskeleton and signaling pathways (*2*). An additional protein translocated into the epithelial cell is the translocated intimin receptor (Tir), which functions as the receptor for the bacterial outer membrane protein, intimin (*3*). EPEC and *C. rodentium* express homologous intimin molecules, intimin α and intimin β, respectively (*4*).

The mechanism by which EPEC causes diarrhea remains unknown, although colonization by EPEC results in increased epithelial permeability to macromolecules (*5*). Live *C. rodentium* expressing intimin β or *C. rodentium* transfected with EPEC intimin α infects the mouse colon and induces severe immunopathology involving mucosal thickening, a $CD4^+$ T cell infiltrate, and a pronounced T helper cell type 1 (T_H1) mucosal immune response that is identical to that seen in mouse models of inflammatory bowel disease (IBD) (*6*). Intimin also binds to β_1-integrins on T cells, the consequence of which is unknown (*7*).

We characterized the consequence of intimin binding to T cells in vitro by examining the effect of the eukaryotic cell–binding, 280–amino acid, COOH-terminal domain of intimin α (int280) (*8*) on submitogenic stimulation of T cells (*9*). When treated with both concanavalin A (Con A) and antibody to CD3 (anti-CD3), purified int280 (*10*) costimulated T cell proliferation in a concentration-dependent manner (Fig. 1A) and increased production of IL-2, IFN-γ, and IL-4 mRNA transcripts (Fig. 1B) (*11*). Int280 alone produced only minor proliferation [two times background counts per minute (cpm)] and no increase in cytokine transcripts (Fig. 1B). The mutation of Cys^{937} of int280 to alanine, which reduces integrin binding (*7*), also reduced costimulatory activity by 54 to 67% (*12*).

To answer the question of whether mucosal immunopathology in live *C. rodentium* infection is a result of epithelial colonization or of intimin interactions with host immune cells, we intracolonically administered formalin-killed bacteria expressing different intimins to BALB/c mice. Wild-type *C. rodentium* expresses intimin β; strain DBS255 is an *eae*-mutant *C. rodentium* that does not express either intimin α or intimin β; strain DBS255 (pCVD438) expresses EPEC intimin α and no intimin β; and strain DBS255 (pCVD438CA) expresses the mutated form of intimin α in which the Cys^{937} is replaced by Ala, thus destabilizing the loop needed for integrin binding but not for Tir binding (*13*).

The epithelial barrier was transiently breached by the intracolonic administration of 50% ethanol (*14*). Mice were then given 4×10^8 of either killed wild-type intimin β expressing *C. rodentium* or intimin$^-$ DBS255 and were killed 6 days later (*14*). Administration of the intimin β^+ killed bacteria recapitulated the immunopathology seen in the live infection (*6*), whereas intimin$^-$ *C. rodentium* had no effect. Macroscopically, distal colons of mice given wild-type *C. rodentium* thickened (Fig. 2A) and were heavier than those of mice given DBS255 (Fig. 2B). Crypts were hyperplastic (Fig. 2C), and there was a predominantly $CD4^+$ and $CD3^+$ T cell and macrophage infiltrate in the lamina propria (*15*) (Fig. 2, D and E). The CD8 response varied between experiments but was always only a minor component of the infiltrate. Mice killed 14 days after administration still had elevated $CD4^+$ and $CD3^+$ infiltrates (*16*). In the tissue, transcripts for TNF-α and IFN-γ and the epithelial growth factor keratinocyte growth factor (KGF) were increased (Fig. 2F). IL-4 transcripts were reduced by 80% (Fig. 2F). These changes are similar to those seen in murine models of IBD (*17*).

Mice receiving dead EPEC intimin α expressing *C. rodentium* (pCVD438) also showed mucosal hyperplasia, with a strong mucosal $CD4^+$ cell response and an increase in T_H1 mRNA transcripts. This effect was markedly reduced when the cysteine-mutated strain (pCVD438CA) was used, although there was a small increase in crypt length and in the number

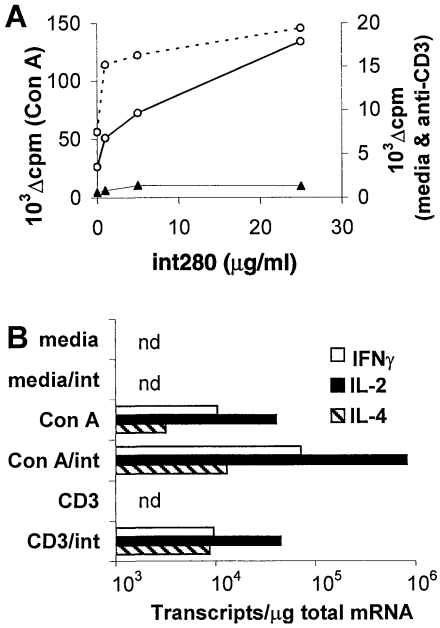

Fig. 1. The effect of int280 on the proliferative response of splenocytes. (**A**) Proliferative response of splenocytes in the presence of int280 (1 to 25 μg/ml) with Con A stimulation (5 μg/ml) (open circle, dashed line), submitogenic anti-CD3 stimulation (145-2C11, 1 μg/ml) (open circle, solid line), or media only (triangle, solid line). (**B**) Cytokine mRNA transcripts in total RNA isolated from cultured cells cultured in media alone, Con A, or anti-CD3, with or without the addition of int280 at 25 μg/ml. Results are representative of two experiments. Background counts per minute were <1000; nd, not detected.

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of infiltrating T cells as compared to the intimin$^-$ control bacterium (Fig. 3, A through D). This is presumably because the CS mutation does not completely abolish intimin binding to β1 integrins. It is also difficult to extrapolate from int280 and int280CS costimulation of T cells in vitro to the in vivo effects of the molecules expressed on the bacterial surface. Mice receiving formalin-killed EPEC strain E2348/69 or a laboratory *E. coli* K12 strain transfected with EPEC intimin α also showed an increase in colon weight and crypt length (Fig. 3, A and B). The in vivo activity of the three different intimin-expressing bacteria suggests that intimin is responsible for inflammation and crypt hyperplasia.

To directly assess the role of IFN-γ in the induction of the intimin-mediated T cell response and colonic hyperplasia, formalin-killed wild-type intimin β$^+$ *C. rodentium* was given to mice that were genetically deficient in the IFN-γ receptor. No immunopathology was observed in these mice as compared to the severe changes seen in the 129Sv$^{+/+}$ controls. Neither colonic weight nor crypt hyperplasia was increased as compared to mice treated with the intimin$^-$ strain (Fig. 4, A and B). A small increase in lamina propria CD4$^+$ cells was seen in these mice (Fig. 4C), but mRNA transcripts for TNF-α, IFN-γ, and KGF were not significantly increased (Fig. 4D).

Thus, intimin is a bifunctional molecule, acting as a ligand for epithelial cell adhesion to Tir in the formation of the AE lesion and also driving mucosal T_H1 responses and subsequent immunopathology. The binding activity of intimin to Tir and β_1 integrins is restricted to the COOH-terminal 280 amino acids (*18*); this domain has two immunoglobulin-like regions and a C-type lectin-like module (*10*), so that there is the potential for interaction with several different receptors.

Although EPEC is traditionally considered a noninvasive pathogen, the high density of bacteria on the epithelial surface means that some bacteria must translocate into the lamina propria. Indeed, in *C. rodentium* infection in mice, bacteria can be seen in the lamina propria and submucosa and there are anecdotal reports of

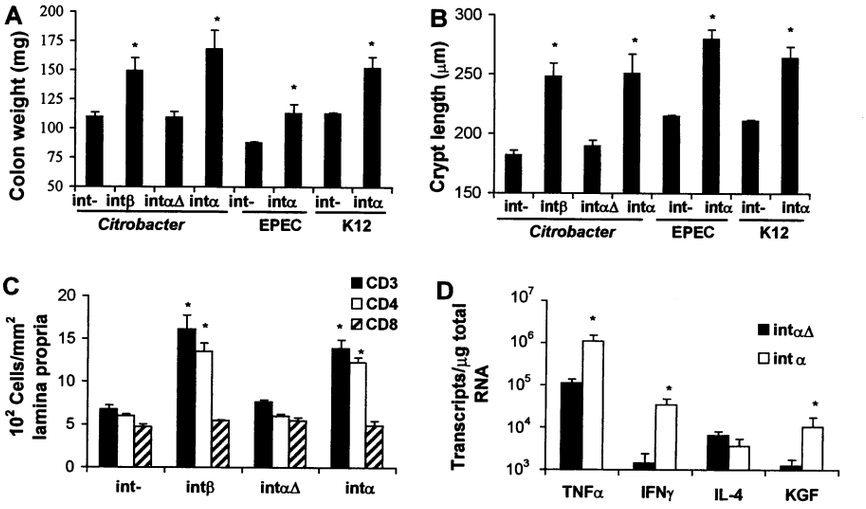

Fig. 3. Intracolonic administration of formalin-killed intimin$^-$ *C. rodentium* DBS255, wild-type intimin β$^+$ *C. rodentium*, intimin α–expressing DBS255(pCVD438), mutant intimin α strain (intαΔ) DBS255(pCVD438CA), intimin α$^+$ EPEC E2348/69, or intimin α$^+$ K12 *E. coli* to BALB/c mice. (**A**) Weight of the distal 4 cm of the colon. (**B**) Crypt length. (**C**) Numbers of CD3$^+$ (black bars), CD4$^+$ (white bars), and CD8$^+$ (hatched bars) cells in the lamina propria. (**D**) mRNA transcripts for TNF-α, IFN-γ, IL-4, and KGF in total RNA isolated from colonic tissue of mice given the DBS255(pCVD438CA) intimin α mutant strain (intαΔ, black bars) or the DBS255(pCVD438) intimin α strain (white bars) [$n = 5$; asterisk indicates $P < 0.05$, Mann-Whitney U test with Bonferroni correction]. All data are shown as the mean ±1 SEM.

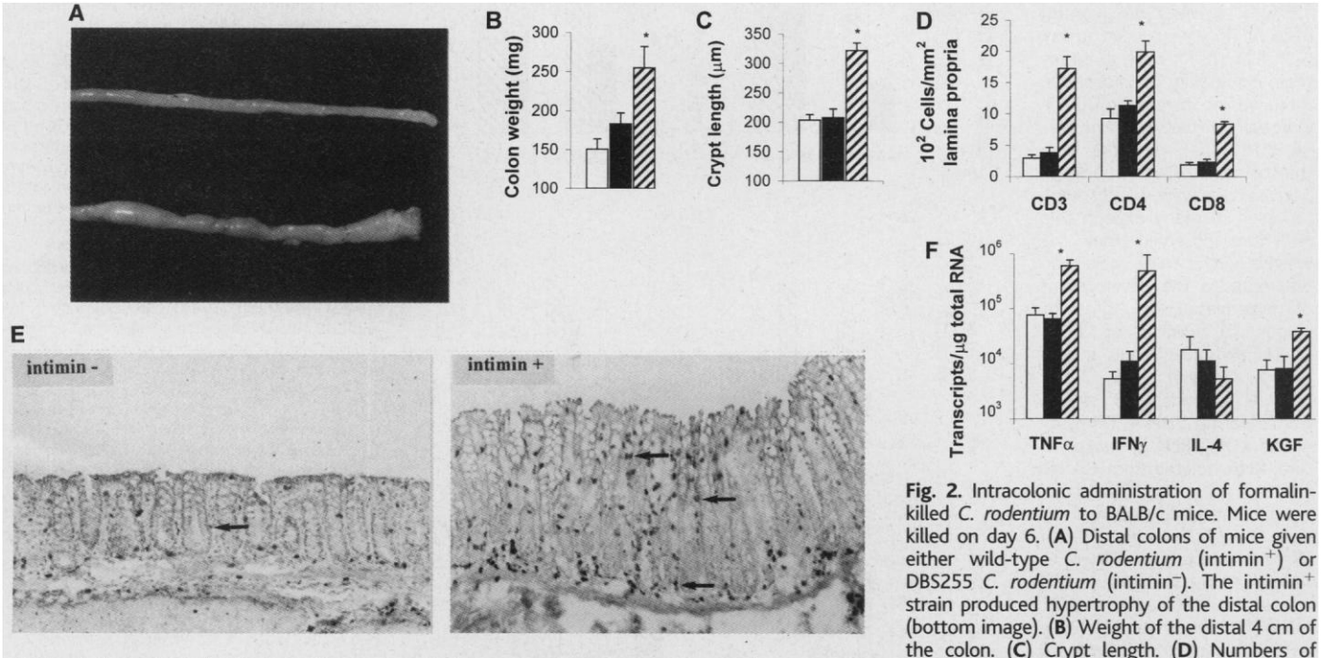

Fig. 2. Intracolonic administration of formalin-killed *C. rodentium* to BALB/c mice. Mice were killed on day 6. (**A**) Distal colons of mice given either wild-type *C. rodentium* (intimin$^+$) or DBS255 *C. rodentium* (intimin$^-$). The intimin$^+$ strain produced hypertrophy of the distal colon (bottom image). (**B**) Weight of the distal 4 cm of the colon. (**C**) Crypt length. (**D**) Numbers of CD3$^+$, CD4$^+$, and CD8$^+$ cells in the lamina propria. (**E**) Immunohistochemistry for CD3$^+$ T cells in the lamina propria of mice given intimin$^-$ or wild-type (intimin$^+$) strains as in Fig. 2A. Positive cells are indicated by arrows (magnification, ×150). (**F**) mRNA transcripts for TNF-α, IFN-γ, IL-4, and KGF. In (B) through (D) and (F), all mice were given 50% ethanol followed by PBS (white bars), the DBS255 intimin$^-$ strain of *C. rodentium* (black bars), or wild-type intimin β$^+$ *C. rodentium* (hatched bars) [$n = 5$; asterisk indicates $P < 0.05$, Mann-Whitney U test with Bonferroni correction]. All data are shown as the mean ±1 SEM.

bacteria in the mucosa in EPEC infection (*6*). Thus intimin has the potential to interact with β_1 integrin–expressing lamina propria T cells (*19*). Int280 alone had very little direct effect on splenic lymphocytes in vitro (Fig. 1A) and is also not directly mitogenic for colonic lamina propria T cells in vitro (*20*), so it is unlikely that it directly activates lamina propria T cells. We were also unable to show that intimin could costimulate resident colonic lamina propria T cells (*20*). However, there are other plausible ways in which intimin can interact with T cells in the gut. The nonspecific recruitment of T cells from the blood into inflamed or infected intestine means that intimin may costimulate naïve T cells responding to antigens from the gut lumen. Alternatively, because physiologically $CD4^+$ T cells respond to gut antigens in the mucosal lymphoid follicles and then migrate to the lamina propria, intimin may be boosting T_H1 responses at the inductive site of mucosal responses rather than at their effector sites in the lamina propria (*20*).

Dead $intimin^+$ *C. rodentium* elicited a strong T_H1 response in the mucosa, and this resulted in mucosal thickening and crypt cell hyperplasia, as seen in mouse models of IBD (*17*). Intimin-induced epithelial proliferation and mucosal thickening at later stages of infection after initial colonization would benefit EPEC and *C. rodentium*. An increased surface area in the crypts would provide a larger area for fresh colonization, and increased epithelial renewal would guarantee a continuing supply of uninfected cells. Increased shedding of colonized enterocytes would increase fecal shedding of the organism and transmission. The increased epithelial renewal in the intestine seen in T cell reactions in the gut has traditionally been considered to be controlled by the host. However, the results presented here strongly suggest that it is citrobacter or EPEC, using intimin, that is specifically driving a mucosal T_H1 response and subsequent immunopathology. The possibility also exists of a bystander effect due to intimin, in that the T_H1 response to other lumenal antigens such as cow's milk proteins or wheat may also be enhanced if antigen exposure in the gut occurs at the same time as EPEC infection.

Many microbial pathogens have evolved sophisticated ways of interacting with host cells and subverting host immune responses by secreting virulence proteins using type III secretion systems. These include, for example, *Salmonella typhimurium* and *Yersinia pseudotuberculosis*, which regulate host cell cytokine signaling pathways through the translocation of proteins into eukaryotic cells (*21*). Our data provide evidence that bacteria have evolved mechanisms to increase immune responses when this would benefit the pathogen.

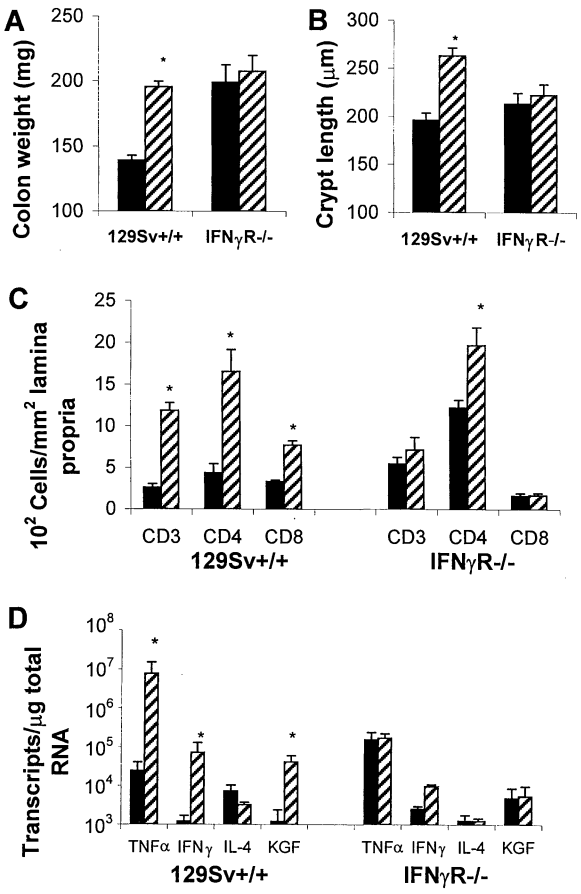

Fig. 4. Intracolonic administration of formalin-killed *C. rodentium* to IFN-$\gamma R^{-/-}$ mice and $129Sv^{+/+}$ wild-type control mice. All mice were killed on day 6. In IFN-$\gamma R^{-/-}$ mice given intimin-expressing *C. rodentium*, there was no increase in (**A**) colonic weight (the weight of IFN-$\gamma R^{-/-}$ colons was more than that of the $129Sv^{+/+}$ controls, because mice were slightly older; however, in many experiments we have observed that the weight of the mice makes no difference to the development of hyperplasia) or (**B**) crypt length. (**C**) Numbers of $CD3^+$, $CD4^+$, and $CD8^+$ cells in the lamina propria of $129Sv^{+/+}$ and IFN-$\gamma R^{-/-}$ mice. (**D**) mRNA transcripts for TNF-α, IFN-γ, IL-4, and KGF mRNA measured in total RNA isolated from colonic tissue. In all mice, 50% ethanol was administered, followed by the DBS255 $intimin^-$ strain (black bars) or by wild-type *C. rodentium* (hatched bars) [$n = 5$; asterisk indicates $P < 0.05$, Mann Whitney U test with Bonferroni correction]. All data are shown as the mean ± 1 SEM.

References and Notes

1. G. Frankel *et al.*, *Mol. Microbiol.* **30**, 911 (1998).
2. B. Kenny, L. C. Lai, B. B. Finlay, M. S. Donnenberg, *ibid.* **20**, 313 (1996); M. S. Donnenberg, J. Yu, J. B. Kaper, *J. Bacteriol.* **175**, 4670 (1993); L. Lai, L. A. Wainwright, K. D. Stone, M. S. Donnenberg, *Infect. Immun.* **65**, 2211 (1997).
3. B. Kenny *et al.*, *Cell* **91**, 511 (1997).
4. J. Adu-Robie *et al.*, *J. Clin. Microbiol.* **36**, 662 (1998).
5. J. Spitz *et al.*, *Am. J. Physiol.* **268**, G374 (1995).
6. L. M. Higgins, G. Frankel, G. Douce, G. Dougan, T. T. MacDonald, *Infect. Immun.* **67**, 3031 (1999).
7. G. Frankel *et al.*, *J. Biol. Chem.* **271**, 20359 (1996).
8. G. Frankel, D. C. A. Candy, P. Everest, G. Dougan. *Infect. Immun.* **62**, 1835 (1994).
9. Single-cell suspensions of splenocytes were plated in 96-well flat-bottomed plates (10^5 per well) in RPMI 1640 medium, supplemented with 10% fetal bovine serum and penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were cultured for 96 hours and were pulsed with ^3H thymidine (1 μCi per well) during the last 15 hours before being harvested onto filters.
10. G. Kelly *et al.*, *Nature Struct. Biol.* **6**, 313 (1999). The purified protein contained no additional bands upon silver staining after SDS–polyacrylamide gel electrophoresis, and electron spray mass spectrometry gave a single mass peak of 30,190 dalton, which is consistent with that predicted from the protein sequence.
11. Total cellular RNA was isolated from gut tissue and cells with guanidine isothiocyanate and phenol. Constructs encoding standard cytokine mRNAs [L. Eckmann, J. Fierer, M. F. Kagnoff. *J. Immunol.* **156**, 2894 (1996)] and a KGF RNA construct [M. Bajaj-Elliott, R. Poulsom, S. L. F. Pender, N. C. Wathen, T. T. MacDonald, *J. Clin. Invest.* **102**, 1473 (1998)] were used for competitive quantitative reverse transcription polymerase chain reaction (RT-PCR). The KGF construct and primers were designed to allow us to detect both mouse and human KGF.
12. Using the same culture conditions as detailed in (9), spleen cells gave a response to CD3 of 6939 ± 915 cpm (background, 1507 ± 808). CD3 plus int280 (5 μg/ml) boosted responses to 16,162 ± 2108, whereas the response to CD3 plus int280CS was only 9853 ± 650.
13. E. L. Hartland *et al.*, *Mol. Microbiol.* **32**, 151 (1999).
14. Bacterial cultures were harvested, resuspended in 10% formalin for 2 hours at room temperature, and washed twice in phosphate-buffered saline (PBS). BALB/c mice (8 to 12 weeks old) were lightly anesthetized with 200 μl of a 1:10 aqueous dilution of Hypnorm. The colonic epithelial barrier was broken by intracolonic administration of 75 μl of 50% ethanol solution [M. F. Neurath, S. Pettersson, K.-H. Meyer Zum Buschenfelde, W. Strober, *Nature Med.* **2**, 998 (1996)]. After 30 min, the same mice received 80 μl of a 5×10^9/ml solution of formalin-killed bacteria intracolonically. Control mice were given PBS.
15. Three-step avidin-peroxidase staining was performed on 5-μm frozen sections using monoclonal antibodies 145-2C11 (anti-CD3), YTS 191 (anti-CD4), and YTS 169 (anti-CD8). Biotin-conjugated rabbit anti-rat immunoglobulin G (IgG) (DAKO, High Wycombe, UK) and goat anti-hamster IgG (Vector Laboratories, Peterborough, UK) were used at a 1:50 dilution in tris-buffered saline (TBS) (pH 7.6) containing 4% (v/v) normal mouse serum (Harlan Seralab, Oxon, UK). Avidin peroxidase (Sigma) was used at a dilution of 1:200 in TBS. Peroxidase activity was detected with 3,3′-diaminobenzidine-tetra-hydrochloride (DAB; Sigma) in tris-HCl (0.5 mg/ml, pH 7.6) containing 0.01% H_2O_2. The density of positive cells in the lamina propria was determined by image analysis.
16. L. M. Higgins and T. T. MacDonald, unpublished data.
17. B. Sadlack *et al.*, *Cell* **75**, 253 (1993); R. Kühn, J. Löhler, D. Rennick, K. Rajewsky, W. Müller, *ibid.*, p. 263; M. W. Leach, A. G. W. Bean, S. Mauze, R. L. Coffman, F. Powrie, *Am. J. Pathol.* **148**, 1503 (1996).
18. G. Frankel *et al. Infect. Immun.* **63**, 4323 (1995).
19. E. C. Ebert and A. I. Roberts, *Cell. Immunol.* **167**, 108 (1996); P. Brandtzaeg, I. N. Farstad, L. Helgeland, in *Chemical Immunology*, T. T. MacDonald, Ed. (Karger, Basel, Switzerland, 1998), vol. 71, p. 1.

20. Resident colonic lamina propria T cells did not respond to CD3 stimulation in vitro nor did they respond to int280 alone over a dose range of 1 to 25 μg/ml, but they did give a proliferative response to Con A (5 μg/ml). This response was not enhanced by int280. However, int280 strongly costimulates T cells from the colonic lymphoid follicles. In response to CD3 activation [details as in (9)], colonic lymphoid follicle T cells gave 9097 ± 1327 cpm, but in the presence of int280 the CD3 response increased to 30,252 ± 4678 cpm.
21. S. Hobbie, L. Chen, R. Davis, J. Galán, *J. Immunol.* **159**, 5550 (1997); K. Schesser *et al.*, *Mol. Microbiol.* **28**, 1067 (1998).

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Genetic Selection of Peptide Inhibitors of Biological Pathways

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Genetic selections were used to find peptides that inhibit biological pathways in budding yeast. The peptides were presented inside cells as peptamers, surface loops on a highly expressed and biologically inert carrier protein, a catalytically inactive derivative of staphylococcal nuclease. Peptamers that inhibited the pheromone signaling pathway, transcriptional silencing, and the spindle checkpoint were isolated. Putative targets for the inhibitors were identified by a combination of two-hybrid analysis and genetic dissection of the target pathways. This analysis identified Ydr517w as a component of the spindle checkpoint and reinforced earlier indications that Ste50 has both positive and negative roles in pheromone signaling. Analysis of transcript arrays showed that the peptamers were highly specific in their effects, which suggests that they may be useful reagents in organisms that lack sophisticated genetics as well as for identifying components of existing biological pathways that are potential targets for drug discovery.

Peptide-protein interactions have critical roles in biology. Many signals are transmitted by the binding of peptides to cell-surface receptors, and many protein-protein interactions inside cells are dominated by the binding of a peptide on one protein to a pocket on another. These interactions have inspired methods to select members of random peptide libraries that bind to known protein targets displayed on the outside of viruses (phage display) (*1*) or within cells (*2*). An alternative strategy is to select peptides whose binding to unknown targets produces a phenotype in the same way that mutations produce phenotypes by inactivating genes (*3*). Like mutations, peptides can be used to probe the function and mechanism of biological pathways as well as to identify their in vivo protein targets.

We developed methods to express peptamers, peptides displayed as an exposed loop on the surface of an inert carrier protein, at high concentrations in budding yeast cells. This approach protects the peptides from proteolytic degradation and imposes some conformational rigidity (*4*). A catalytically inactive version of staphylococcal nuclease (*5*) was used as a carrier protein because it is small, folds spontaneously without chaperones, has a prominently exposed loop on its surface (*6*), and can be strongly expressed as a soluble protein in eukaryotes and prokaryotes. The peptamer libraries contained 16 random amino acids inserted into the staphylococcal nuclease open reading frame (ORF) in place of the carrier's most exposed surface loop (*7*).

Because the extent of pathway inhibition depends on inhibitor concentration, we maximized expression of the peptamers. A high-copy vector was made that contains a strong constitutive promoter driving the expression of a staphylococcal nuclease gene that uses optimal codons for efficient translation and epitope tags for immunological detection and protein purification (*8*). Cells containing this vector expressed the peptamers as one of the most abundant proteins in the cell (Fig. 1).

We developed selections for inhibitors of two signal transduction pathways, the spindle checkpoint (*9*) and the mating pheromone response pathway (*10*). The spindle checkpoint arrests cells in mitosis in response to chromosomes that fail to attach to the mitotic spindle (*11*) and the pheromone pathway arrests cells in G_1 in response to a peptide mating factor. Both pathways are good targets for inhibitor selection because neither is essential for viability, and activation of either pathway prevents cell proliferation, creating a selection for peptamers that inhibit the pathway.

The spindle checkpoint is evolutionarily conserved and is defective in many human tumor cell lines (*12*). Selecting for inhibitors of the spindle checkpoint requires genetic trickery. In normal cells, the checkpoint is activated by improperly aligned chromosomes, and overriding the checkpoint in these cells leads to errors in chromosome segregation and cell death (*13*). However, overexpression of the checkpoint protein Mps1 activates the checkpoint in cells that have normal spindles (*14*). In this situation, inactivating the checkpoint allows cells to divide and form viable colonies. Thus, we engineered the selection strain to overexpress Mps1 when grown on galactose (*15*). We identified inhibitors of the spindle checkpoint by transforming the peptamer library into this strain and selecting for the rare transformants that formed colonies on galactose-containing medium (*16*). From a pool of 6.5×10^6 transformants, we identified three peptamers that allow cells to proliferate on galactose (Fig. 2A). Two of the

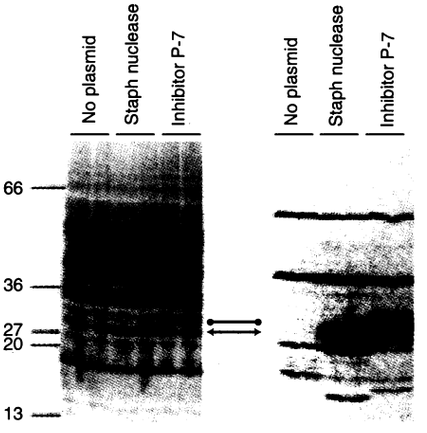

Fig. 1. Peptamer expression in yeast. Measurement of peptamer expression in yeast by Coomassie staining (left) and antihemagglutinin immunoblotting (right). Double-headed arrow denotes staphylococcal nuclease; double-headed closed circle denotes peptamer P-7. Numbers on left are kilodaltons.

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