

achieve a productive and substrate-specific catalytic complex, and this induced-fit mechanism provides a direct opportunity for allosteric regulation. In analogy to an allosteric dimer, the active site of AC is formed at the interface between dyad-related homologous domains. Thus, AC provides two symmetrically related binding sites for the homologous G proteins G_{α} and G_{β} (4, 5). We suggest that G_{α} facilitates collapse of the active-site loops from C_{1a} and C_{2a} around the substrate ATP to form an competent active site, whereas G_{β} hinders such collapse and stabilizes an open, inactive conformation of the enzyme.

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- the presence of Mg^{2+} alone or of Mn^{2+} and Mg^{2+} , and for the entire triphosphate in the presence of Zn^{2+} and Mg^{2+} . Much weaker density corresponding to a purine ring is observed in the purine binding pocket of these complexes. However, the purine and triphosphate densities are not continuous, and a single model of ATP cannot be built to accommodate both purine and triphosphate binding sites.
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- We thank F. Eckstein at the Max-Planck Institute (Göttingen) for pure $ATP\alpha S-R_p$; J. Collins for technical assistance; C. Brautigam for helpful discussion and for reading the manuscript; D. Coleman and the MacCHESS staff for their assistance with data collection at the Cornell High Energy Synchrotron Source (CHESS); and L. Esser for his assistance in preparing figures. R.K.S. was supported by a postdoctoral fellowship from the Medical Research Council of Canada. This work was supported by grants from the Agence Nationale de Recherche sur le SIDA (ANRS, France) to G.G.; by NIH grants DK38828 (R.A.J.), GM34497 (A.G.G.), and DK46371 (S.R.S.); by an Innovative Technology Grant from the Stony Brook Center for Biotechnology to R.A.J.; by Welch Foundation grants I-1271 (A.G.G.) and I-1229 (S.R.S.); and by the Raymond and Ellen Willie Distinguished Chair of Molecular Neuropharmacology (A.G.G.). Coordinates for the four models have been deposited in the Protein Data Bank with the codes 1CJT, 1CJU, 1CJV, and 1CJL.

26 April 1999; accepted 22 June 1999

Staphylococcus aureus Sortase, an Enzyme that Anchors Surface Proteins to the Cell Wall

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Surface proteins of Gram-positive bacteria are linked to the bacterial cell wall by a mechanism that involves cleavage of a conserved Leu-Pro-X-Thr-Gly (LPXTG) motif and that occurs during assembly of the peptidoglycan cell wall. A *Staphylococcus aureus* mutant defective in the anchoring of surface proteins was isolated and shown to carry a mutation in the *srtA* gene. Overexpression of *srtA* increased the rate of surface protein anchoring, and homologs of *srtA* were found in other pathogenic Gram-positive bacteria. The protein specified by *srtA*, sortase, may be a useful target for the development of new antimicrobial drugs.

Hospital isolates of *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Enterococcus faecalis* have become resistant to most, if not

all, known therapeutic regimens (1). Many antibiotics, including penicillin and its derivatives, target the transpeptidation reaction of bacterial cell wall synthesis, which cross-links peptidoglycan strands (2). To search for other cell wall synthesis reactions that may serve as targets for antimicrobial therapy, we have focused on the anchoring of surface proteins to the peptidoglycan of Gram-positive bacteria.

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REPORTS

Surface proteins not only promote interaction between the invading pathogen and animal tissues, but also provide ingenious strategies for bacterial escape from the host's immune response (3). In the case of *S. aureus* protein A, immunoglobulins are captured on the microbial surface and camouflage bacteria during the invasion of host tissues (4). Protein A is cleaved by a transpeptidase, sortase, between the threonine and the glycine of a conserved LPXTG motif (5). The carboxyl group of threonine

is amide-linked to the amino group of the pentaglycine cross-bridge, thereby tethering the COOH-terminal end of protein A to the bacterial cell wall (6). This reaction, called cell wall sorting, is strikingly similar to the penicillin-sensitive transpeptidation reaction, and is likely to occur in most Gram-positive bacteria (7).

To identify cell wall sorting mutants, we mutagenized *S. aureus* strain OS2 with nitrosoguanidine (8). Temperature-sensitive (ts) mutants were identified and 1000 were trans-

formed with pSEB-SPA₄₉₀₋₅₂₄, a plasmid encoding a reporter protein that allows measurement of surface protein anchoring (9). The SEB-SPA₄₉₀₋₅₂₄ precursor (P1) is exported from the cytoplasm, and its NH₂-terminal leader peptide is removed to generate the P2 intermediate (Fig. 1A) (10). P2 is cleaved by sortase at the LPXTG motif to generate the mature, surface-anchored protein (M). After labeling with [³⁵S]Met for 5 min, the reporter protein in strain OS2 was distributed as follows: P1 (5%), P2 (19%), and M (76%) (Fig. 1B) (11). We used this assay to screen 1000 ts mutants and identified two strains in which there was aberrant accumulation of P2 (47% in SM317 and 26% in SM329) (Fig. 1B). Pulse-chase analysis (12) revealed that in strain OS2, P2 was cleaved and anchored within 2 min, whereas in strain SM317 these events required more than 10 min (Fig. 1C). In strain SM329, P2 processing required 3 min, suggesting a mild defect in cell wall sorting (13).

Previous work showed that mutations in *S. aureus fem* genes slow the anchoring of surface proteins to the cell wall (14). These genes are thought to specify enzymes that catalyze the addition of glycines to the ε-amino of lysine within the peptidoglycan precursor lipid II (15). To examine whether the SM317 and SM329 mutants were defective in cell wall synthesis, we tested their sensitivity to lysostaphin, an enzyme that cuts the pentaglycine cross-bridges of the staphy-

Fig. 1. Isolation of a staphylococcal mutant defective in cell wall sorting of surface proteins. (A) Primary structure of the surface protein precursor SEB-SPA₄₉₀₋₅₂₄, a fusion between enterotoxin B (SEB) and COOH-terminal protein A (SPA) sequences. P1 is directed across the cytoplasmic membrane by an NH₂-terminal leader peptide, and is then cleaved to generate P2. P2 bears a COOH-terminal sorting signal that includes an LPXTG motif, a hydrophobic domain (black bar), and a positively charged tail (boxed +). The sorting signal of P2 is cleaved at the LPXTG motif, and the mature protein (M) is linked to the cell wall. (B) *Staphylococcus aureus* ts mutants were screened for the accumulation of P2 by labeling with [³⁵S]Met. SM317 and SM329 accumulate more P2 than does the wild-type (WT) strain OS2. (C) Pulse-chase analysis of SEB-SPA₄₉₀₋₅₂₄ anchoring in wild-type and mutant strains.

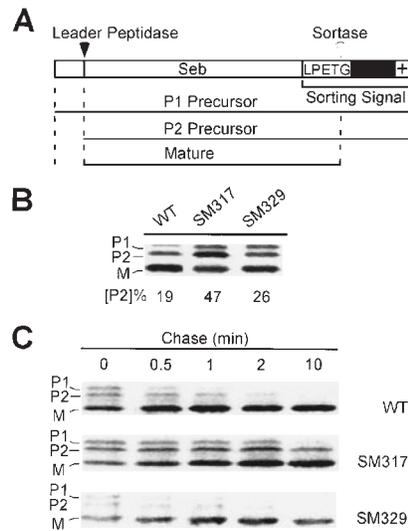


Fig. 2. Structure of surface protein anchor in strain SM317. (A) Primary structure of SEB-MH₆-CWS and its linkage to the cell wall. The glycan strands of the staphylococcal cell wall consist of a repeating disaccharide *N*-acetylmuramic acid-(β1-4)-*N*-acetylglucosamine (MN-GN) in which the lactyl of muramic acid is linked to the wall peptide (L-Ala-D-iGln-L-Lys-D-Ala). Wall peptides are linked to the pentaglycine cross-bridge, which tethers the ε-amino of L-Lys to the carboxyl of D-Ala. The cell wall can be cut at specific sites with the enzymes muramidase (solid arrow) and φ11 hydrolase (open arrows). (B) SEB-MH₆-CWS was solubilized by digesting the staphylococcal cell wall with muramidase (mutanolysin) and purified by affinity chromatography on Ni-NTA resin. The polypeptide was cleaved with cyanogen bromide (CnBr), and COOH-terminal anchor peptides were purified by another affinity chromatography step. The structure of the mutanolysin-released anchor peptides of strain SM317 was analyzed by MALDI-MS (6). (C) Muramidase-released anchor peptides were digested with φ11 hydrolase and analyzed by MALDI-MS. The observed ions represent anchor peptide linked to cell wall tetrapeptide (*m/z* 2236) and murein-disaccharide tetrapeptide (*m/z* 2715 and 2757).

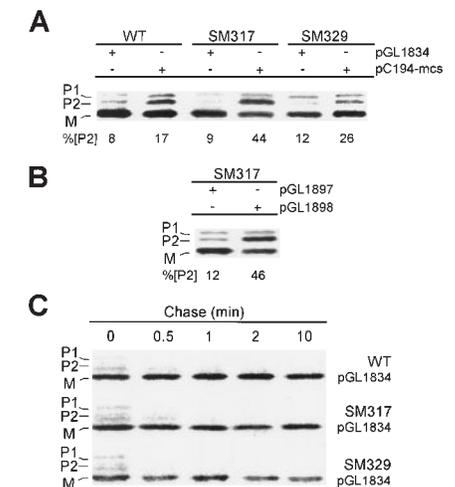
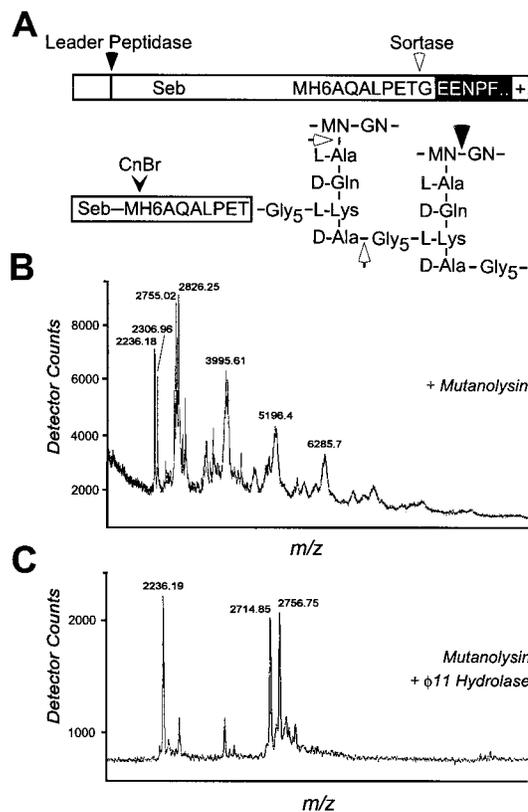


Fig. 3. Overexpression of *srtA* reduces P2 accumulation in wild-type *S. aureus* strain OS2 and in two mutant strains, SM317 and SM329. (A) Transformants of a multicopy plasmid library in strain SM317 were screened by labeling with [³⁵S]Met for a decrease in the accumulation of P2. Plasmid pGL1834 contains the *srtA* gene cloned into pC194-mcs. (B) The *srtA* gene of strain SM317 (pGL1898) or of strain OS2 (pGL1897) was transformed into SM317 and analyzed for P2 processing. (C) All three strains were transformed with pGL1834 and subjected to pulse-chase analysis (12).

lococcal cell wall (16). In contrast to *fem* mutants, which are resistant to lysostaphin (17), strains SM317 and SM329 were sensitive at concentrations that also inhibited growth of wild-type staphylococci, indicating that their sorting defects are not caused by a mutationally altered cell wall cross-bridge (18). To measure cell wall synthesis, we grew the wild-type and mutant strains in minimal medium containing [³H]Lys or [³H]Leu. Because lysine (but not leucine) is a component of the cell wall, the ratio of [³H]Lys:[³H]Leu incorporation into acid-precipitable and protease-resistant murein polymer is a measure of cell wall synthesis (19). Wild-type *S. aureus* displayed a ratio of 30, and the inhibition of cell wall synthesis by vancomycin reduced this ratio to 1.5. Strains SM317 and SM329 displayed ratios of 18 and 19, respectively, which indicates that the accumulation of P2 in strain SM317 is not caused by a defect in cell wall synthesis.

To determine the cell wall anchor structure of surface proteins in strain SM317, we introduced into cells a plasmid (pHTT4) specifying the reporter protein SEB-MH₆-CWS (Fig. 2A) (6). The cell wall was purified and digested with mutanolysin, which hydrolyzes the glycan strands (6). Mutanolysin-released surface protein was purified by chromatography on nickel-nitrilotriacetic acid (Ni-NTA) and cleaved at Met with cyanogen bromide. COOH-terminal peptides bearing cell wall anchor structures were purified by a second affinity chromatography step and analyzed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) (Fig. 2). A series of ion signals with regularly spaced mass increments was revealed. These measurements are consistent with increasing numbers of peptidoglycan subunits linked to the COOH-terminal threonine of surface protein (20). If surface protein is tethered to cross-linked peptidoglycan of strain SM317, digestion of muramidase-solubilized anchor peptides with ϕ 11 hydrolase should produce anchor peptide linked to murein tetrapeptide and disaccharide-tetrapeptide (21) (Fig. 2). This was tested, and the doubly digested anchor peptides generated the predicted ion signals (Fig. 2C). Thus, surface proteins of *S. aureus* SM317 are tethered to cross-linked peptidoglycan in a manner that is indistinguishable from the anchor structure of polypeptides in wild-type staphylococci.

These results suggest that the accumulation of P2 in strain SM317 is caused by a defect in sortase.

We reasoned that overexpression of sortase from a multicopy plasmid should reduce the concentration of P2 in both wild-type and mutant *S. aureus*. A plasmid library of 2000 random DNA fragments from *S. aureus* OS2 was screened for sequences that reduce the accumulation of P2 in strain SM317 and two plasmids, pGL1631 and pGL1834, were identified (Fig. 3) (22). Transformation with pGL1834 reduced the P2 concentration in strain SM317 by 35%, in strain SM329 by 14%, and in wild-type *S. aureus* OS2 by 9%. All three strains showed a rapid increase in P2 processing (Fig. 3C). We mapped the critical *S. aureus* sequences in pGL1631 and pGL1834 to a gene that we named *srtA* (surface protein sorting A) (18, 23).

The *srtA* gene specifies a protein of 206 amino acids with a potential NH₂-terminal signal peptide/membrane anchor sequence and a presumed active-site cysteine at position 184, consistent with the observation that the cell wall sorting reaction is sensitive to reagents that modify sulfhydryl groups (Fig. 4) (10). Database searches revealed that *srtA* homologs are present in *Actinomyces naeslundii*, *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*. All *srtA* homologs displayed absolute conservation of the cysteine-encoding codon 184 (18).

To examine whether the defect in cell wall sorting of *S. aureus* SM317 is caused by a *srtA* mutation, we amplified the genes from *S. aureus* OS2 and SM317 by PCR, and cloned them into a multicopy vector, which was then transformed into *S. aureus* SM317 (18, 23). The wild-type (OS2) *srtA* gene reduced the accumulation of P2 by 32%, whereas the mutant had no effect (Fig. 3B). DNA sequence analysis revealed mutations in codons 35 and 180 in the SM317 *srtA* gene. Interestingly, multicopy expression of wild-type *srtA* (pGL1894) did not fully complement the ts growth phenotype of SM317. The transformed mutant grew at 42°C but slower than the wild type, suggesting that the conditional lethal phenotype of *S. aureus* SM317 is not caused solely by the mutations in *srtA*.

Together, our data reveal that *srtA* encodes sortase, the transpeptidase that anchors surface proteins to the bacterial cell wall

(24). In principle, purified SrtA protein can be used to screen for compounds that inhibit cell wall sorting, a strategy that may lead to new therapies for human infections caused by Gram-positive bacteria.

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8. Staphylococci (10¹² colony-forming units) were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (0.2 mg/ml) for 45 min at 30°C, and mutagenesis was quenched by the addition of 2 volumes of 100 mM sodium phosphate (pH 7.0). About 80% of the mutagenized population was killed, and the mutational frequency of rifampicin-resistant *rpoB* mutations was increased to 1.2 × 10⁻⁴. Ts mutants were selected three times in succession by growing the mutagenized population in tryptic soy broth (TSB) at 42°C and treating with penicillin G (8 µg/ml) for 2 hours. Colonies were formed at 30°C, streaked on TSB agar, and examined for growth at 42°C.
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Fig. 4. Deduced amino acid sequence of the *srtA* gene (25). The NH₂-terminal hydrophobic membrane anchor sequence is boxed. The single Cys is shaded. The *srtA* gene of strain SM317 carries two mutations, one in codon 35 replacing Asp (GAT) with Gly (GGT), and another in codon 180 replacing Thr (ACA) with Ala (AGA). The altered amino acids are indicated in bold. The DNA sequence of plasmid pGL1834 has been submitted to GenBank (accession number AF162687).

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MKKWTNRIMT IAGVVLILVA AYLFAKPHID 30
NYLHDKDKDE KIEQYDKNVK EQASKDKKQQ 60
AKPQIPKDKS KVAGYIEIPD ADIKEPVYPG 90
PATPEQLNRG VSFAEENESL DDQNSIAGH 120
TFIDRPNYQF TNLKAAKKS MVYFKVGNET 150
RKYKMTSIRD VKPTDVGVLD EQKGKDKQLT 180
LITQDDYNEK TGVWEKRKIF VATEVK* 206
    
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- (predicted mass 2755, observed 2755), N,O6-diacetyl-MurNac-GlcNac pentapeptide (predicted mass 2828, observed 2826), murein-tetrapeptide-murein-pentapeptide (predicted mass 3990, observed 3995), (murein-tetrapeptide)₂-murein-pentapeptide (predicted mass 5194; observed 5196), and (murein-tetrapeptide)₄ (predicted mass 6285, observed 6286).
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 22. Plasmid pGL4 contains the coding sequence of SEB-SPA₄₉₀₋₅₂₄ which was released from pSEB-SPA₄₉₀₋₅₂₄ by Eco RI-Bam HI digestion and inserted into the pT181 derivative pWil5. *S. aureus* SM317 (pGL4) was grown on TSB tet agar. A plasmid library of *S. aureus* OS2 chromosomal DNA was obtained by partial digestion with Sau 3A1. DNA fragments of 3 to 5 kb were purified and
 - cloned into Bam HI-digested pC194-mcs, which contains the multiple cloning site of pUC19 inserted into the Hind III site of pC194. SM317 (pGL4) was transformed with the pC194-mcs plasmid library and transformants were selected on TSB tet-cm agar.
 23. The DNA insertions of pGL1631 and 1834 were mapped and sequenced by synthesizing oligonucleotide primers. The primers for the amplification of *srtA* from the chromosomal DNA of *S. aureus* strains OS2 (pGL1897) and SM317 (pGL1898) were 5'-AAG-GATCCAAAGGAGCGGTATACATTGC-3' and 5'-AAGGATCCTACCTTTCTCTAGCTGAAG-3'.
 24. In another report we show that purified SrtA protein catalyzes the in vitro transpeptidation of substrates bearing an LPXTG motif (H. Ton-That, G. Liu, S. K. Mazmanian, K. F. Faull, O. Schneewind, in preparation).

25. Abbreviations for the amino acid residues are as follows: 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.
26. We thank W. W. Navarre for help in determining the surface protein anchor structure of strain SM317, and D. Missiakas, P. Model, M. Russel, and members of our laboratory for critical reading of this manuscript. S.K.M. was supported by the Predoctoral Training Program in Genetic Mechanisms at UCLA (T32GM07104). H.T.-T. was supported by the Microbial Pathogenesis Training Grant at UCLA (AI 07323). Work in O.S.'s laboratory is supported by grant AI33987 from the National Institute of Allergy and Infectious Diseases.

6 May 1999; accepted 28 June 1999

Phosphorylation and Sequestration of Serotonin Transporters Differentially Modulated by Psychostimulants

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Many psychotropic drugs interfere with the reuptake of dopamine, norepinephrine, and serotonin. Transport capacity is regulated by kinase-linked pathways, particularly those involving protein kinase C (PKC), resulting in transporter phosphorylation and sequestration. Phosphorylation and sequestration of the serotonin transporter (SERT) were substantially impacted by ligand occupancy. Ligands that can permeate the transporter, such as serotonin or the amphetamines, prevented PKC-dependent SERT phosphorylation. Nontransported SERT antagonists such as cocaine and antidepressants were permissive for SERT phosphorylation but blocked serotonin effects. PKC-dependent SERT sequestration was also blocked by serotonin. These findings reveal activity-dependent modulation of neurotransmitter reuptake and identify previously unknown consequences of amphetamine, cocaine, and antidepressant action.

Serotonin [5-hydroxytryptamine (5-HT)] is a platelet-stored vasoconstrictor that also acts as a transmitter in the nervous system to modulate a wide spectrum of behaviors (1). The actions of 5-HT are terminated by active transport (2). Whereas 5-HT actions are mediated by >15 different types of receptors, a single 5-HT transporter (SERT) is responsible for extracellular 5-HT clearance (3). SERT activity is blocked by cocaine and tricyclic antidepressants. Serotonin-selective reuptake inhibitors (SSRIs) like fluoxetine (Prozac) preferentially block SERTs and enhance serotonergic signaling in affective disorders (2, 4). The amphetamines are substrates for SERTs, as well as for dopamine (DA) and norepinephrine (NE) transporters (DATs and NETs, respectively) (5) and can trigger SERT-mediated release of 5-HT (5–8). Repeated administration of amphetamines

sensitizes monoaminergic synapses to subsequent psychostimulant challenge (9), which may involve modulated protein kinase cascades (10). Alterations in SERT activity and binding site density (11) and SERT gene polymorphisms (12) have implicated the transporter in anxiety, depression, suicide, autism, and substance abuse. Recent findings with transgenic mice (13) support an important role for 5-HT and SERTs in the behavioral actions of cocaine and amphetamine.

SERT expression can be rapidly modulated by receptor stimulation, second messenger production, and kinase activation (14–16). Suppression of SERT activity accompanying protein kinase C (PKC) activation (17) arises from a loss of 5-HT uptake capacity (V_{max}). The loss in 5-HT uptake capacity correlates with a loss of surface-expressed SERTs (17), similar to the PKC modulation of homologous γ -aminobutyric acid (GABA), DA, and NE transporters (18). PKC activators and phosphatase inhibitors induce SERT phosphorylation (19) with a similar time course and kinase antagonist sensitivity as observed for changes in 5-HT transport.

We investigated whether the regulation of SERTs was influenced by transport and whether SERT ligands differentially influenced SERT regulation. Figure 1A shows that PKC-mediated SERT phosphorylation in transfected human embryonic kidney-293 (HEK-293) cells was substantially diminished if assayed in the presence of the transported neurotransmitter, 5-HT. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of immunoprecipitates from [³²P]PO₄-labeled cell extracts (20) revealed a three- to fivefold stimulation of human SERT (hSERT) phosphorylation after phorbol 12-myristate 13-acetate (β -PMA) application. This stimulation was abolished by coapplication of PKC antagonists. In the presence of 5-HT (1 μ M), SERT phosphorylation triggered by phorbol esters was also substantially blunted. At low concentrations of β -PMA (for example, 10 nM), 5-HT essentially abolished phorbol ester-induced SERT labeling. At 200 nM β -PMA, where labeling of SERTs is maximal, we consistently achieved 40 to 60% inhibition of SERT phosphorylation at maximal concentrations of 5-HT (1 μ M) with a median effective concentration (EC₅₀) of 70 nM (Fig. 1B).

If the actions of 5-HT on SERT phosphorylation arise as a consequence of transport, then an intrinsic homeostatic loop might be present to link transporter expression to extracellular amine availability, and incubation with SERT antagonists should block this effect. Indeed, the SERT-selective tricyclic antidepressant imipramine (1 μ M) or the SSRIs paroxetine (1 μ M) and citalopram (1 μ M) blocked the ability of 5-HT to limit PKC-dependent SERT phosphorylation (Fig. 1C). This effect was selective for SERT antagonists, as neither the DAT inhibitor GBR-12909 nor the NET antagonist nisoxetine could affect the ability of 5-HT to blunt SERT phosphorylation (Fig. 1C). There are no known 5-HT receptor subtypes on HEK-293 cells, and to our knowledge, 5-HT does not induce acute changes in cyclic adenosine 5'-monophosphate, inositol trisphosphate, or intracellular Ca²⁺ levels in these cells. Moreover, the ability of 5-HT to diminish PKC-dependent SERT phosphorylation was not

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***Staphylococcus aureus* Sortase, an Enzyme that Anchors Surface Proteins to the Cell Wall**

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Science **285** (5428), 760-763.
DOI: 10.1126/science.285.5428.760

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