Structure of the Cell Wall Anchor of Surface Proteins in *Staphylococcus aureus*

Olaf Schneewind,* Audree Fowler, Kym F. Faull

Many surface proteins are anchored to the cell wall of Gram-positive bacteria and are involved in the pathogenesis of these organisms. A hybrid molecule was designed that, when expressed in *Staphylococcus aureus*, was anchored to the cell wall and could be released by controlled enzymatic digestion. By a combination of molecular biology and mass spectrometry techniques, the structure of the cell wall anchor of surface proteins in *S. aureus* was revealed. After cleavage of surface proteins between threonine and glycine of the conserved LPXTG motif, the carboxyl of threonine is amide-linked to the free amino group of the pentaglycine crossbridge in the staphylococcal cell wall.

Human infections caused by Gram-positive bacteria present an increasing therapeutic challenge because of the recent appearance of antibiotic-resistant strains (*1*). Proteins displayed on the surface of Gram-positive bacteria contribute in many different ways to the pathogenesis of these infections (*2*). For example, surface proteins of the Gram-positive organism *Staphylococcus aureus* interact with specific host molecules, thereby either concealing the bacterial surface from the defense system or promoting adhesion of the pathogen to host tissues (*3*).

Staphylococcal protein A binds avidly to the complement-fixing part of immunoglobulins that cover the bacterial surface during infection (*4*). The NH_2-terminal immunoglobulin-binding domains of protein A are displayed on the cell surface, whereas the COOH-terminal end is anchored to the bacterial cell wall (*5–7*). This ability to anchor to the cell wall requires a 35-residue sorting signal that is located at the predicted COOH-terminus of protein A (*7*) and consists of an LPXTG motif, followed by a COOH-terminal hydrophobic domain and a tail of mostly positively charged residues (*8*). Homologous sequences have been found in surface proteins of many different Gram-positive species (*9*), and several of these sequences also function to anchor fusion proteins to the bacterial cell wall (*8*). Cell wall–anchored molecules of Gram-positive bacteria have similar topologies in that the NH_2-terminal domains are displayed on the cell surface, whereas the COOH-terminal anchor structures are buried in the thick peptidoglycan layer where it has been difficult to elucidate their structure (*10*).

To identify the chemical linkage between surface proteins and the staphylococcal cell wall, we designed a hybrid molecule (Fig. 1A). Our experimental scheme relies on the fact that maltose binding protein (MalE) of *Escherichia coli* is an exportable and protease-resistant molecule (*11*), which allows for the specific removal and characterization of NH_2- and COOH-terminal fusion peptides by means of engineered trypsin cleavage sites. The mature part of MalE was fused between NH_2-terminal staphylococcal enterotoxin B sequences, including the signal (leader) peptide as well as the six additional residues ESQPVP, and the COOH-terminal cell wall sorting signal of protein A (*12*). When expressed in *S. aureus*, this hybrid protein (MalE-Cws) was anchored to the cell wall and could only be solubilized by enzymatic digestion of the peptidoglycan. Muramidase cleavage of the glycan strands in the staphylococcal cell wall (*13*) solubilized MalE-Cws as a spectrum of fragments with increasing mass as a result of a variable number of linked cell wall units (*8*). In contrast, lysostaphin cut the cell wall close to the anchoring point of surface proteins at the pentaglycine crossbridge (*14*) and released MalE-Cws as a single uniform species of 43 kD that migrated faster on SDS–polyacrylamide gel electrophoresis (PAGE) than the muramidase-solubilized counterparts (Fig. 1B).

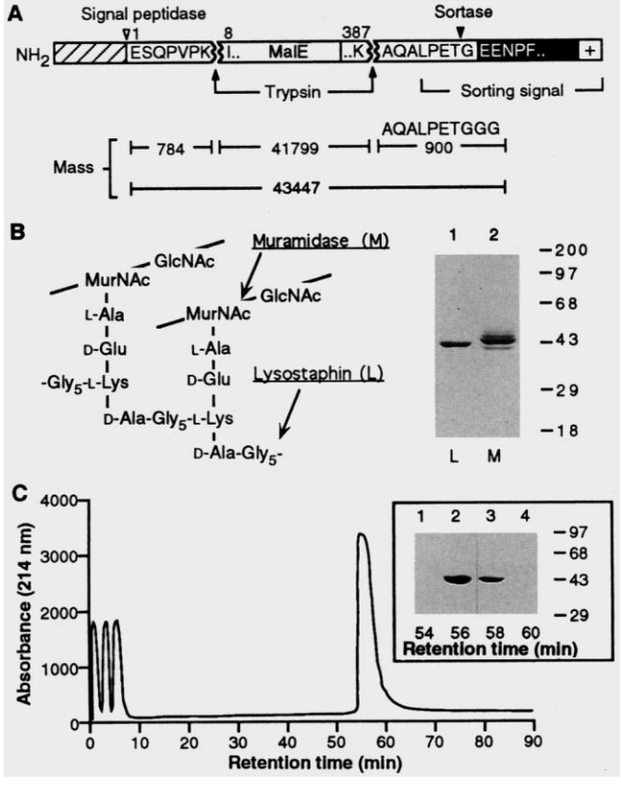

Fig. 1. (**A**) Deduced primary structure of cell wall–anchored MalE-Cws. The hybrid protein consists of the mature maltose binding protein (MalE) fused between the NH_2-terminal signal peptide (including six residues of mature enterotoxin B) (hatched bar, residues 1 to 6) and the sorting signal plus 13 upstream residues of protein A (sequences downstream of the crossbar following MalE). The black bar and the boxed (+) represent the COOH-terminal hydrophobic domain and the charged tail of the cell wall sorting signal, respectively. Letters indicate amino acid residues of the NH_2-terminal (K at position 7) and COOH-terminal (K at position 387) trypsin cleavage sites and their surrounding sequences. Note that the predicted amino acid sequence following the LPXTG motif of the sorting signal of MalE-Cws (LPETGEENPF..) differs from that determined for the cell wall–linked molecule (LPETGGG). The signal peptidase (open triangle) and sortase (closed triangle) cleavage sites are indicated. The experimentally determined molecular masses for the lysostaphin-solubilized MalE-Cws molecule and for its trypsin cleavage fragments (arrows) are displayed. The scale of the drawings is not proportional to the length or mass of the protein sequences. (**B**) Structure of the cell wall of *S. aureus* and of the cleavage sites for lysostaphin (L) and muramidase (M) (*25*). GlcNAc, *N*-acetylglucosamine; MurNAc, *N*-acetylmuramic acid. The Coomassie-stained SDS-PAGE (10%) shows MalE-Cws purified after lysostaphin (lane 1) or muramidase (lane 2) solubilization of the staphylococcal cell wall. The molecular sizes of standard proteins are indicated to the right in kilodaltons. (**C**) An rpHPLC chromatogram of affinity-purified MalE-Cws; the inset shows the Coomassie-stained SDS-PAGE (10%) of aliquots of HPLC fractions at retention times of 54 (lane 1), 56 (lane 2), 58 (lane 3), and 60 (lane 4) min. The molecular sizes of standard proteins are indicated to the right in kilodaltons.

O. Schneewind, Department of Microbiology and Immunology and Molecular Biology Institute, University of California School of Medicine, Los Angeles, CA 90024, USA.
A. Fowler, Department of Biological Chemistry and Molecular Biology Institute, University of California School of Medicine, Los Angeles, CA 90024, USA.
K. F. Faull, Department of Psychiatry and Biobehavioral Sciences and the Neuropsychiatric Institute, University of California School of Medicine, Los Angeles, CA 90024, USA.

*To whom correspondence should be addressed.

We purified lysostaphin-released MalE-Cws by affinity chromatography and reversed-phase high-performance liquid chromatography (rpHPLC) (Fig. 1C) (*15*). Purified MalE-Cws was subjected to Edman degradation, which confirmed that the signal (leader) sequence had been cleaved. Electrospray mass spectrometry of MalE-Cws gave a mass of 43446.96 with an estimated experimental error of ±4 (0.01%). This mass cannot be explained on the basis of the predicted protein sequence of the hybrid molecule. The result must be accounted for by the mass of the MalE-Cws protein plus the cell wall component to which it is linked.

Trypsin-treated MalE-Cws migrated faster on SDS-PAGE than the untreated counterpart (Fig. 2A), indicating that part of the hybrid protein was sensitive to proteolysis (*16*). Sequencing of the NH_2-terminus revealed that trypsin treatment had removed seven residues, ESQPVPK, from MalE-Cws. After the trypsin-treated MalE-Cws molecule was purified by rpHPLC (Fig. 2B), its mass was determined to be 41,799.51 (Fig. 3B). This observation is consistent with the calculated mass of the polypeptide chain spanning residues 8 to 387 (41,797.55), indicating that trypsin cleavage also occurred near the COOH-terminal end of MalE-Cws, three residues upstream of the LPXTG motif after Lys^{387} (Fig. 1A).

Subtraction of the mass of trypsin-treated MalE-Cws (41,799) from that of the untreated molecule (43,447) yields the combined mass of the NH_2- and COOH-terminal cleavage products after addition of two water molecules that are required for proteolysis [(43,447 + 36 = 43,483) – 41,799 = 1684]. Subtracting the known mass of the NH_2-terminal tryptic fragment (784) from the combined mass suggests a mass of 900 for the COOH-terminal peptide containing the cell wall anchor (1684 – 784 = 900). We have previously shown that during cell wall anchoring of surface proteins the sorting signal is proteolytically cleaved between the threonine and the glycine of the LPXTG motif (*17*). The fact that lysostaphin cleavage of the pentaglycine crossbridge solubilized MalE-Cws as a single uniform species rather than as a spectrum of differently sized fragments suggests that this crossbridge is directly involved in the anchor structure of surface proteins. The free amino group of the pentaglycine crossbridge could form an amide bond with the carboxyl of threonine, thus anchoring surface proteins. We therefore asked how many glycines linked to the threonyl of the LPXTG motif would give a mass of 900 and found one combination with three linked glycine residues and a mass of 900.44 (AQALPETGGG).

We used liquid chromatography mass spectrometry (LC-MS) to scan portions of pooled HPLC fractions of trypsin-treated MalE-Cws for the NH_2- and COOH-terminal cleavage fragments, and peptides with masses of 784.3 and 900.5 were identified in the first pooled fraction (*18*) (Fig. 2B). The remainder of this sample was therefore purified by rpHPLC, and the collected fractions were again analyzed by electrospray mass spectrometry (*18*) (Fig. 2C). The two peptides with masses of 784.3 and 900.5 were identified in separate fractions, both of which were subjected to Edman degradation (Fig. 3). The sequence of the peptide

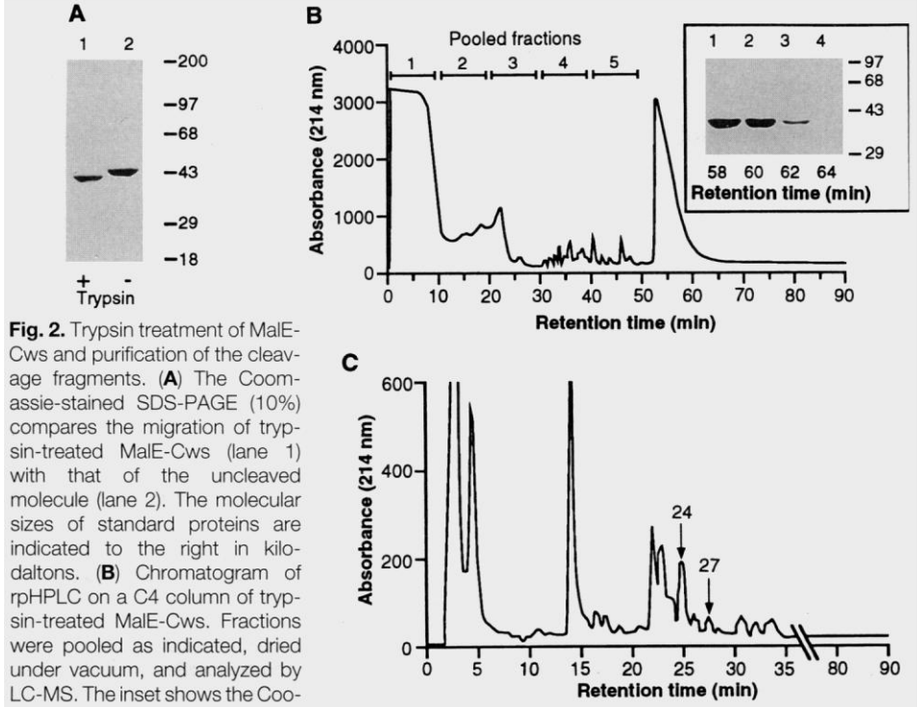

Fig. 2. Trypsin treatment of MalE-Cws and purification of the cleavage fragments. (**A**) The Coomassie-stained SDS-PAGE (10%) compares the migration of trypsin-treated MalE-Cws (lane 1) with that of the uncleaved molecule (lane 2). The molecular sizes of standard proteins are indicated to the right in kilodaltons. (**B**) Chromatogram of rpHPLC on a C4 column of trypsin-treated MalE-Cws. Fractions were pooled as indicated, dried under vacuum, and analyzed by LC-MS. The inset shows the Coomassie-stained SDS-PAGE (10%) of aliquots of HPLC fractions containing trypsin-truncated MalE-Cws at retention times of 58 (lane 1), 60 (lane 2), 62 (lane 3), and 64 (lane 4) min. The molecular sizes of standard proteins are indicated to the right in kilodaltons. (**C**) Chromatogram of rpHPLC on a C18 column of pooled fraction 1 from (B) containing the NH_2- and COOH-terminal tryptic peptides. Fractions corresponding to individual chromatographic peaks were analyzed by mass spectrometry, and the arrows indicate peaks containing the NH_2-terminal peptide of mass 784.3 (fraction 24) and the COOH-terminal peptide of mass 900.5 (fraction 27).

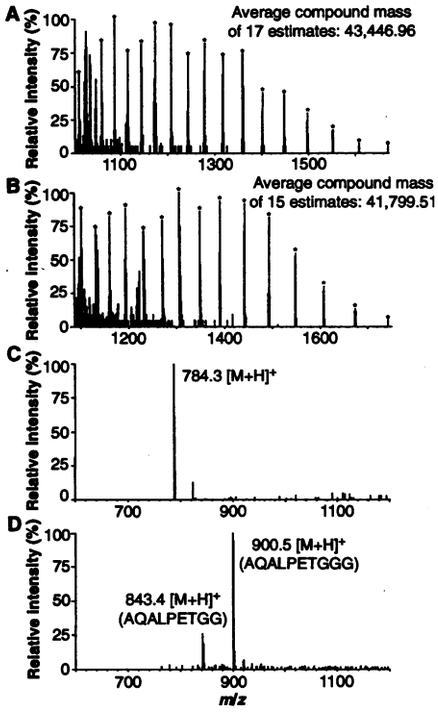

Fig. 3. Typical electrospray mass spectra for MalE-Cws and its trypsin cleavage fragments. Stars indicate measurements used for average mass calculations. (**A**) MalE-Cws lysostaphin-solubilized from the staphylococcal cell wall; the calculated average mass for a molecule with three glycines amide-linked to the threonyl of the LPXTG motif is 43,445.36, and the observed average mass is 43,446.96. (**B**) Trypsin-cleaved MalE-Cws spanning residues 8 to 387; the calculated average mass is 41,797.55, and the observed average mass is 41,799.51. (**C**) NH_2-terminal trypsin cleavage fragment of MalE-Cws (ESQPVPK); the calculated monoisotopic mass is 784.42 (protonated form), and the observed mass is 784.3. (**D**) COOH-terminal trypsin cleavage fragment of MalE-Cws (AQALPETGGG); the calculated monoisotopic mass is 900.44 (protonated form), and the observed mass is 900.5. A second ion of mass 843.4 corresponds to the calculated mass of a peptide with the sequence AQALP ETGG (843.42, protonated form).

with mass 784.3 was ESQPVPK, corresponding to the NH_2-terminal protein sequence of MalE-Cws. The sequence AQALPETGG(G) was determined for the COOH-terminal peptide with a mass of 900.5 (*19*). Electrospray mass spectrometry of this sample revealed two ions: a strong signal at a mass-to-charge ratio (m/z) of 900.5 (AQALPETGGG) and a second weaker ion at m/z 843.4, an observation which is consistent with the calculated mass of the peptide sequence AQALPETGG (Fig. 3D). These results can be explained either by the presence of two different peptides with similar chromatographic behavior in the same sample or by the fragmentation of the larger peptide with mass 900.5 (AQALPETGGG) during mass spectrometry. The latter explanation is more likely because the mass measurements of lysostaphin-solubilized MalE-Cws are consistent with the presence of three glycines amide-linked to the threonyl of the LPXTG motif (Fig. 3).

When tested with a synthetic pentaglycine peptide, lysostaphin cleaves randomly between any of the four glycyl-glycine peptide bonds (*20*); however, the same may not be true when purified staphylococcal peptidoglycan is used as a substrate (*21*). Mass measurements of MalE-Cws and of its purified COOH-terminal peptides indicate that lysostaphin cleavage occurred almost exclusively between the third and fourth glycine of the pentaglycine crossbridge, a selectivity which could be the result of steric hindrances imposed by the proximity of both the anchored surface protein and the linked cell wall peptide.

Our data demonstrate that MalE-Cws is linked to the staphylococcal cell wall through an amide bond between the COOH-terminal carboxyl of threonine and the amino of the pentaglycine crossbridge. Conservation of both the LPXTG motif in sorting signals (*9*) and the free amino groups in peptidoglycan crossbridges (*13*) suggests that cell wall sorting in Gram-positive bacteria occurs by a general mechanism. A model for an amide bond exchange mechanism in which surface proteins are linked to the crossbridges of several different bacterial peptidoglycans is proposed in Fig. 4. Surface proteins are first exported by means of an NH_2-terminal signal (leader) peptide. Secretion of the polypeptide chain into the surrounding medium is prevented by a COOH-terminal retention signal, which consists of several positively charged residues (*8*). Retention is followed by the proteolytic cleavage of surface proteins between threonine and glycine of the LPXTG motif (*17*). The carboxyl of threonine is subsequently amide-linked to the free amino of the peptidoglycan crossbridge, pentaglycine in *S. aureus*.

Although peptidoglycan crossbridges display much chemical variation among Gram-positive bacteria, the presence of a free amino group is a common feature (*22*). As a result of extensive cross-linking by means of transpeptidation (*23*), assembled staphylococcal peptidoglycan contains few free amino groups (*24*). Therefore, it is likely that surface proteins are linked to the free amino groups of the pentaglycine crossbridge before the final cross-linking is completed (*25*). Although cell wall anchoring of surface proteins in staphylococci is extremely efficient (*8*), some of these molecules can also be found in culture supernatants (*26*). This phenomenon may be caused by the physiological turnover and release of peptidoglycan fragments with linked surface proteins into the culture medium (*27*). The enzyme responsible for the cell wall linkage of surface proteins in Gram-positive bacteria has not been thus far identified, but its specific inhibition could represent a novel target for antibacterial therapy.

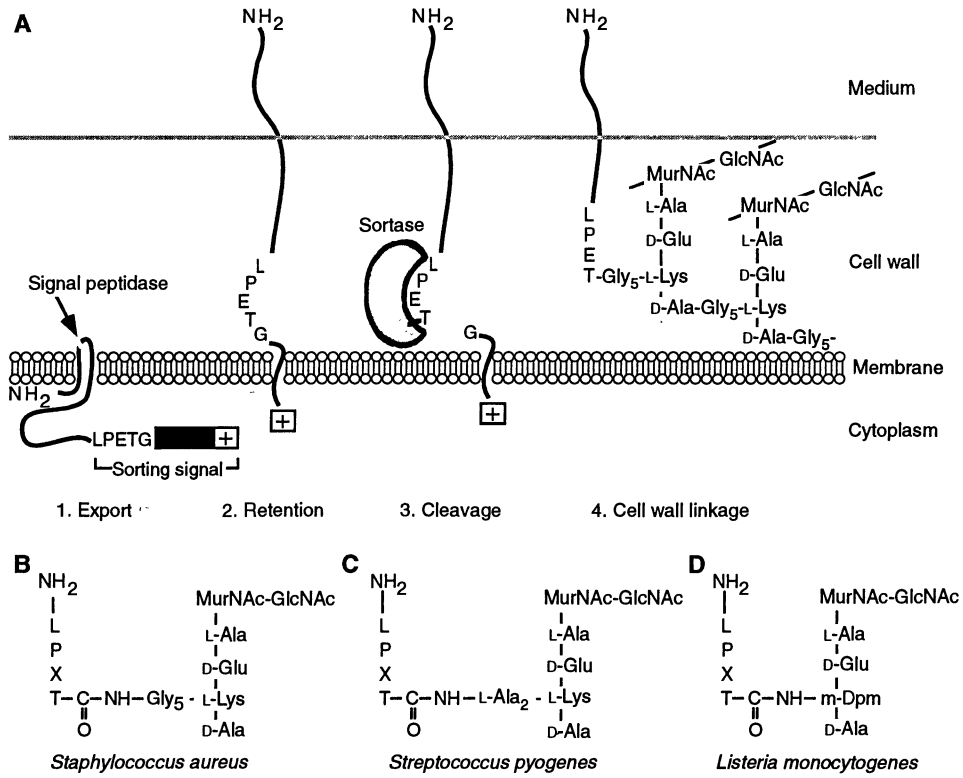

Fig. 4. Diagram showing the structure of the cell wall anchor of surface proteins in *S. aureus* and in other Gram-positive bacteria. (**A**) Cell wall sorting of surface proteins consists of four distinct steps, which lead to the proteolytic cleavage of the polypeptide chain between threonine and glycine of the LPXTG motif. The carboxyl of threonine is subsequently amide-linked to the free amino group in the pentaglycine crossbridge of the staphylcoccal cell wall (*24, 25*). The cell wall linkage of surface proteins in *S. aureus* (**B**) is compared with that proposed for other Gram-positive bacteria such as *Streptococcus pyogenes* (**C**) and *Listeria monocytogenes* (**D**).

REFERENCES AND NOTES

1. B. R. Lyon and R. Skurray, *Microbiol. Rev.* **51**, 88 (1987); H. C. Neu, *Science* **257**, 1064 (1992); C. T. Walsh, *ibid.* **261**, 308 (1993).
2. M. A. Kehoe, in *Bacterial Cell Wall,* J.-M. Ghuysen and R. Hakenbeck, Eds. (Elsevier, Amsterdam, 1994), pp. 217–261.
3. T. J. Foster and D. McDevitt, *FEMS Microbiol. Lett.* **118**, 199 (1994).
4. T. Moks *et al.*, *Eur. J. Biochem.* **156**, 3577 (1986); A. H. Patel, P. Nowlan, E. D. Weavers, T. Foster, *Infect. Immun.* **55**, 3103 (1987).
5. J. Sjöquist, J. Movitz, I.-B. Johansson, H. Hjelm, *Eur. J. Biochem.* **30**, 190 (1972).
6. J. Sjöquist, B. Meloun, H. Hjelm, *ibid.* **29**, 572 (1972).
7. O. Schneewind, P. Model, V. A. Fischetti, *Cell* **70**, 267 (1992).
8. O. Schneewind, D. Mihaylova-Petkov, P. Model, *EMBO J.* **12**, 4803 (1993). Abbreviations for the amino acid residues are A, Ala; C, Cys; E, Glu; F, Phe; G, Gly; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; S, Ser; T, Thr; and V, Val.
9. V. A. Fischetti, V. Pancholi, O. Schneewind, *Mol. Microbiol.* **4**, 1603 (1990).
10. E. Munõz, J.-M. Ghuysen, H. Heymann, *Biochemistry* **6**, 3659 (1967); B. Guss *et al.*, *Eur. J. Biochem.* **138**, 413 (1984); V. Pancholi and V. A. Fischetti, *J. Bacteriol.* **170**, 2618 (1988); *J. Exp. Med.* **170**, 2119 (1989).
11. L. L. Randall, *Cell* **33**, 231 (1983).
12. The coding sequences of *malE* [P. Duplay *et al.*, *J. Biol. Chem.* **259**, 10606 (1984)] were amplified from *E. coli* K38 chromosomal DNA [A. Garen and N. D. Zinder, *Virology* **1**, 347 (1955)] with the oligonucleotides 5′-AAAAGGTACCCAAAATCGAAGAAGGTAAACTGGT-3′ and 5′-AAAAGCTTGGTGATACGAGTCTGCGCGT-3′. Sequences coding for the sorting signal of protein A were amplified from the chromosomal DNA of *S. aureus* 8325-4 [M. Uhlén *et al.*, *J. Biol. Chem.* **259**, 1695 (1984)] with primers 5′-AAAAGCTTCAACCAGCAAACCATGCAGA-3′ and 5′-AAGGATCCTTATCATTTCAAATAAGAATGTGTT-3′.

The promoter and signal sequence of staphylococcal enterotoxin B [C. L. Jones and S. A. Khan, *J. Bacteriol.* **166**, 29 (1986)] were amplified from the chromosomal DNA of *S. aureus* S6 with oligonucleotides 5′-AAGAATTCGTATATAAGTTTAGGTGATGT-3′ and 5′-AAGGTACCGGTTGACTCTCTGCTAAAA-3′. The following genes were digested with the indicated restriction enzymes: *seb*, Eco RI and Kpn I; *malE*, Kpn I and Hind III; and *spa*, Hind III and Bam HI. The resulting DNA fragments were cloned between the Eco RI and Bam HI sites of pOS1 (*8*). The recombinant plasmid pSebsp–MalE-Cws was transformed into *S. aureus* OS2 (*7*) and maintained by chloramphenicol selection. The hybrid protein consists of the first 33 residues of Seb, which includes the signal peptide and six residues of the mature sequence fused to the NH_2-terminal lysine of mature MalE. The sorting signal and 13 upstream residues of protein A are fused to the COOH-terminal lysine of MalE with a leucine linker preceding Glu^{477} of protein A. The open reading frame of MalE-Cws was sequenced, revealing a single point mutation that changed Gly^{306} to aspartic acid (G998A transition).

13. J.-M. Ghuysen, *Bacteriol. Rev.* **32**, 425 (1968).
14. C. A. Schindler and V. T. Schuhardt, *Proc. Natl. Acad. Sci. U.S.A.* **51**, 414 (1964).
15. For a typical purification 10^{12} colony-forming units of an exponential *S. aureus* OS2 (pSebsp–MalE-Cws) culture were harvested by 10,000*g* centrifugation from 4 liters of tryptic soy broth. Cells were resuspended in 200 ml of water and extracted with 200 ml of ice-cold acetone:ethanol (1:1) for 15 min [R. P. Novick, *Methods Enzymol.* **204**, 587 (1991)]. After the addition of another 400 ml of water, the cells were washed in 800 ml of STM buffer (0.5 M sucrose, 50 mM tris-HCl, pH 8.0, and 10 mM $MgCl_2$), resuspended in 140 ml of prewarmed STM, and digested with 10 mg of lysostaphin (Applied Microbiology) for 2 hours at 37°C. Protoplasts were collected by 20,000*g* centrifugation for 30 min, and the supernatant (cell wall digest) was filtered, dialyzed against TSM buffer (20 mM tris-HCl, pH 7.5, 200 mM NaCl, and 10 mM mercaptoethanol), and loaded onto 10 ml of amylose resin [T. Ferenci and U. Klotz, *FEBS Lett.* **94**, 213 (1978)] (New England Biolabs). The column was washed with 200 ml of TSM, and MalE-Cws was finally eluted with 10 ml of 10 mM maltose in TSM. The eluate was dialyzed against 50 mM NH_4HCO_3, concentrated by filtration on centriprep-30/centricon-30 membranes (Amikon), and the protein concentration determined by comparison with a standard (MBP-2**, New England Biolabs). A typical purification yielded 0.1 mg of pure MalE-Cws. Purified MalE–Cws (1 μg) was separated on a 10% SDS-PAGE, electroblotted onto Trans-Blot membrane (Bio-Rad), stained (0.25% Brilliant Blue R-250 in 40% methanol), and the excised band subjected to Edman degradation. The NH_2-terminal sequence ESQPVPKI was determined, and reactions were stopped after eight cycles. For rpHPLC purification, 0.3 mg of purified protein was injected (3× 250 μl in 50 mM NH_4HCO_3, 20% glacial acetic acid) onto a rpHPLC column [2.1 mm by 250 mm Shandon Hypersil butyl, 5-μm particle size, eluted with an increasing concentration of acetonitrile, 1% per minute in 0.1% trifluoroacetic acid (TFA)]. MalE-Cws eluted at 56% acetonitrile, 0.1% TFA; the corresponding fraction was dried under vacuum and solubilized in 50 μl of 50% acetonitrile 0.1% formic acid, and 5 μl (100 pmol) were injected into the ion source of an electrospray mass spectrometer (Sciex API III R) and scanned at *m/z* 1000 to 1800. About 40 scans were averaged, and signals were mass measured by means of the multiply charged ion series from the separate introduction of polypropylene glycol for calibration. Deconvolution of the series of multiply charged ions and calculation of protein molecular weight were achieved with the Hypermass computer program. Under standard conditions the accuracy of these mass measurements is 0.01% of the molecular weight. For muramidase digestion, the acetone-ethanol extracted cells were resuspended in STM buffer, pH 6.8, and digested with 10,000 units of mutanolysin [K. Yokogawa, S. Kawata, S. Nishimura, Y. Ikeda, Y. Yoshimura, *Antimicrob. Agents Chemother.* **6**, 156 (1974)] and 100 mg of egg white lysozyme (Sigma) for 12 hours at 37°C. Mutanolysin (Sigma) contains a substantial protease contamination which was inhibited with 2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) 5 min before the addition of the enzyme to the cells.
16. The optimal temperature and enzyme-to-substrate ratio for tryptic digestion of MalE-Cws were established: 0.3 mg of affinity-purified MalE-Cws was digested with 11 μg of sequencing-grade modified trypsin (Promega) at 25°C for 2 hours in 500 μl of 50 mM NH_4HCO_3. The digestion was stopped by the addition of 120 μl of glacial acetic acid, and portions (2 μg) were removed for SDS-PAGE analysis. After electroblotting of the SDS-PAGE and staining with Brilliant Blue, the bands were excised and analyzed by NH_2-terminal sequencing which yielded the sequence IEEGKLV. The remainder of the trypsin digest was subjected to rpHPLC (*15*). The large MalE-Cws cleavage product eluted at 58% acetonitrile. The corresponding fraction was dried under vacuum, solubilized, and mass measured (*15*).
17. W. W. Navarre and O. Schneewind, *Mol. Microbiol.* **14**, 115 (1994).
18. The 2-min fractions 1 to 5, 6 to 10, 11 to 15, 16 to 20, and 21 to 25 of the rpHPLC run of trypsin-digested MalE-Cws (*16*) were pooled, dried under vacuum, and resuspended in 50 μl of 0.1% TFA. Samples (5 μl each) were injected onto rpHPLC column (Keystone Scientific Betasil C18, 1 mm by 100 mm, 5-μm particle size, 100 Å pore size, linear gradient of acetonitrile 1% per minute in 0.1% TFA), and the effluent was passed directly into the electrospray mass spectrometer. The NH_2- and COOH-terminal peptides with mass 784.3 and 900.5, respectively, were identified in the pooled fractions 1 to 5 and eluted at 25% (NH_2-terminal peptide) or 26% (COOH-terminal peptide) acetonitrile. The remainder of pooled fraction 1 (45 μl) was subjected to rpHPLC (2 mm by 250 mm Shandon Hypersil ODS, 3-μm particle size, linear gradient of acetonitrile 1% per minute in 0.1% TFA); the peptides of interest eluted at 24% (NH_2-terminal peptide) and 27% acetonitrile (COOH-terminal peptide).
19. As determined with a Porton 2090E sequencer, the amounts of amino acids recovered after Edman degradation of the COOH-terminal peptide were 714 pmol (A), 584 pmol (Q), 430 pmol (A), 262 pmol (L), 104 pmol (P), 91 pmol (E), 33 pmol (T), 27 pmol (G), 27 pmol (G), and 23 pmol (G). The yield of amino acids declined rapidly after the sixth cycle [after the cleavage of glutamic acid (E) in the COOH-terminal peptide AQALPETGGG], presumably because the remaining peptides were retained with decreased efficacy on the sequencing filter.
20. H. P. Browder, W.A. Zygmunt, J. R. Young, P. A. Tavoramina, *Biochem. Biophys. Res. Commun.* **19**, 383 (1965); G. L. Sloan, E.C. Smith, J. H. Lancaster, *Biochem. J.* **167**, 293 (1977); P. A. Recsei, A. D. Gruss, R. P. Novick, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1127 (1987).
21. B. L. M. de Jonge, Y.-S. Chang, D. Gage, A. Tomasz, *J. Biol. Chem.* **267**, 11248 (1992).
22. K. H. Schleifer and O. Kandler, *Bacteriol. Rev.* **36**, 407 (1972).
23. D. J. Tipper and J. L. Strominger, *Proc. Natl. Acad. Sci. U.S.A.* **54**, 1133 (1965).
24. J.-M. Ghuysen, D. J. Tipper, C. H. Birge, J. L. Strominger, *Biochemistry* **4**, 2245 (1965); J.-F. Petit, E. Munöz, J.-M. Ghuysen, *ibid.* **5**, 2765 (1966).
25. J. L. Strominger, K. Izaki, M. Matsuhashi, D. J. Tipper, *Fed. Proc.* **26**, 9 (1967); J. L. Strominger and J.-M. Ghuysen, *Science* **156**, 213 (1967).
26. A. Forsgren, *Acta Pathol. Microbiol. Scand.* **75**, 481 (1969).
27. G. D. Shockman and J. F. Barrett, *Annu. Rev. Microbiol.* **37**, 501 (1983).
28. We thank D. Boyd, S. Daefler, P. Model, and M. Russel for many discussions and critically reading the manuscript. We also thank C. Canzanella, W. W. Navarre, and T. Baba for critical comments on the manuscript. We acknowledge the technical assistance of J. Chuang and P. Abdul-Sayed during these studies. Supported by NIH grant AI 33985 to O.S.

7 October 1994; accepted 21 December 1994

Antigen-Specific Development of Primary and Memory T Cells in Vivo

Michael G. McHeyzer-Williams and Mark M. Davis

The expansion and contraction of specific helper T cells in the draining lymph nodes of normal mice after injection with antigen was followed. T cell receptors from purified primary and memory responder cells had highly restricted junctional regions, indicating antigen-driven selection. Selection for homogeneity in the length of the third complementarity-determining region (CDR3) occurs before selection for some of the characteristic amino acids, indicating the importance of this parameter in T cell receptor recognition. Ultimately, particular T cell receptor sequences come to predominate in the secondary response and others disappear, showing the selective preservation or expansion of specific T cell clones.

Antigen-specific primary and memory helper T cell responses are central to the establishment of protective immunity (*1*). Tracking the fate of these antigen-specific helper T cells in vivo has been a significant technical problem, mainly because of their very low frequencies in normal animals. Whereas ligand binding can be monitored directly in the B cell compartment (*2*), this has not been possible for T cells because of their relatively low affinities (*3*). To overcome this problem, we used the mouse T cell antigen pigeon cytochrome c (PCC), which is restricted by $I\text{-}E^k$ (*4*). T cells that respond to this antigen express a uniform type of αβ T cell receptor (TCR) heterodimer ($V_\alpha 11V_\beta 3$) (*5*). By staining cells with antibodies specific for these V regions

M. G. McHeyzer-Williams, Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA 94305, USA.
M. M. Davis, Department of Microbiology and Immunology and Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA 94305, USA.