Bacterial lipoproteins contain a lipid-modified Cys as the first amino acid of the mature protein and are synthesized as precursors with a consensus of Leu-Ala/Ser-Gly/Ala-Cys at the cleavage site (11). The sequence specificity for prolipoprotein modification and signal peptide cleavage by signal peptidase II has been investigated in detail elsewhere (10, 16, 27, 37). These studies have demonstrated a requirement for the consensus cleavage site and in addition have shown that the termination of the central hydrophobic region of the signal sequence is important for efficient processing. The signal sequence itself is rapidly degraded, and as a result, few studies of sequence specificity for this degradation have been done, although a number of proteases of Escherichia coli which are capable of signal peptide hydrolysis have been identified (15, 25, 26). An exception to this rapid degradation exists in the colicin lysis proteins (or bacteriocin release proteins), which are required for the release of colicins from E. coli cells (8). A number of these small membrane lipoproteins are processed unusually slowly, releasing stable signal peptides that accumulate in the cytoplasmic membrane (4, 13, 22). Experiments in which all but the last residue of the signal sequence of the cloacin DF13 lysis protein were replaced by that of the major lipoprotein of E. coli (or Braun’s lipoprotein) showed that these properties are due to the signal peptide itself rather than the mature portion of the protein (23). A number of studies have also examined the possible role of the stable signal peptide itself in colicin release and in the partial lysis and cell death (quasilysis) which occur as the colicin is released from induced cultures. In studies of the processing and degradation of the cloacin DF13 lysis protein signal peptide, a lysis protein synthesized with the unstable signal peptide of Braun’s lipoprotein did not function in cloacin release but did cause lethality and quasilysis (23). Expression of the stable signal peptide alone caused lethality and quasilysis but no cloacin release, as did a number of hybrid lysis protein-major lipoprotein signal peptides of varying stability (33, 35). In addition, when the stable signal peptide and the inactive lysis protein synthesized with an unstable signal peptide were expressed together, a small amount of cloacin DF13 was released from the cells (34). Kanoh et al. constructed a mutant colicin E2 lysis protein which was composed of essentially only the signal peptide and showed that this provoked cell death but was inactive in colicin release (20). These results have suggested that the lysis protein signal peptides may play a mechanistic role in quasilysis and the release of colicins from producing cells, perhaps through formation of a pore composed of the peptides and the mature lysis protein (23, 33, 35).

In the studies described here, we have mutated specific amino acids of the colicin A lysis protein (Cal) signal sequence, which resulted in much faster processing, or both faster processing and destabilization of the signal peptide, depending on the mutation. These results indicated that the Ile residue at position 13 of this signal peptide is chiefly responsible for its stability, while Ile13 and Ala18 both contribute to the slow modification and processing of the precursor. Colicin secretion studies with these mutants also demonstrated that neither the accumulation of processing intermediates nor the accumulation of stable signal peptides is required for either colicin release or the quasilysis and cell death caused by this lysis protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. E. coli W3110 and W3110 depP were used as the hosts for the induction experiments, while HB101 was used as the host for all recombinant constructions. Cultures used for [35S]methionine labeling experiments were grown at 37°C in M9 medium supplemented with 0.5% methionine assay medium, 4 mg of glycerol per ml, 10 μg of thiamine per ml, and 50 μg of ampicillin per ml, while those labeled with [3H]glycerol were grown in the same medium supplemented with 4 mg of sodium lactate per ml rather than glycerol as the carbon source. For quasilysis and colicin secretion assays, cultures were grown in Luria-Bertani medium (24). The source of the cal gene for the in vitro mutagenesis experiments was the colicin A plasmid pK9, a derivative of...
pBR322 containing the entire colicin A operon under the control of its natural SOS promoter (12, 21).

Construction of cal mutations. PCR was used as previously described (32) to create the Ala18—Gly18 and Ile13—Ser13 mutations in the cal gene of plasmid pKA. The derivative pKAN was first constructed by using oligonucleotides PH4 (5′ 5ACTTTTACGAAAAAGTAAAG) and PH3 (5′ GACTTGGCTAGCCG ACTAGCAT) to introduce a silent T→A mutation at codon 16 of the cal gene, creating an NheI site. This allowed oligonucleotides PH4 and PH7 (5′ TGGCCCTGACCACTGAGCAGCG) to be used to change the Ile13 codon (ATG) to Ser (TCC) in pKANS13 and oligonucleotides PH6 (5′ TGTPTGCTAGCCGATGAGCAGCG) and PH5 (5′ CTCCTGCGGGATGATCAGGAG) to be used to change the Ala18 codon (GCA) to the Gly codon (GGA) in pKANG18. These mutations were then combined by exchanging an NheI-HindIII fragment from pKANG18 containing the Ala18—Gly18 mutation into pKANS13 to yield pKANS13G18. The structure of each of the mutant cal genes was verified with PH4 and PH1, which hybridize 5′ and 3′ to the cal gene, respectively, as the sequencing primers (28). pBR22 derivatives of each of the plasmids containing the cal mutations were constructed by digestion with SfiI and NcoI, treatment with the Klenow fragment of DNA polymerase I, and religation of the plasmid as previously described (2).

Labeling and immunoprecipitation experiments. W3110 cells containing the various plasmids were induced with 300 ng of metionine C per ml and labeled with 11.5 Ci/mmol after 45 min of induction as previously described (12), except that the radioactive concentration was 1 mCi/ml. After various periods of chase with 2.5 mg of unlabeled metionine per ml, 10-μl samples were withdrawn from the cultures and mixed with an equal volume of immunoprecipitation buffer containing 2% sodium dodecyl sulfate (SDS), boiled to a red, and left for 30 min with immunoprecipitation buffer containing 1% Triton X-100, as described by Ito et al. (19). The monoclonal antibody CA1, coupled to Affi-Gel HZ (Bio-Rad), was added in excess (as determined in preliminary experiments), and after overnight incubation at 4°C, the immunoprecipitates were washed three times before being resuspended in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and electrophoresed (19). For lipoprotein labeling in the presence or absence of globomycin, W3110 degP cells were used as the host in order to reduce the degradation of pCal which occurs in the presence of this drug (5). The cells were induced with 300 ng of metionine C per ml, and when labeled, globomycin was added to a concentration of 100 μg/ml after a further 10 min. After an additional 10 min of incubation, [2-3H]glycerol (200 μCi/ml, 11.5 Ci/mmol) was used to pulse the cells for 30 min, after which they were immunoprecipitated as described above.

Other methods. SDS-PAGE was performed in a Tricine buffer system (29). Autoradiograms of SDS-PAGE gels of these immunoprecipitates indicated that only pCal immunoprecipitates were separated by SDS-PAGE and visualized by autoradiography. The positions of migration of pCal (C), pCalm (●) (which comigrates with the major lipoprotein in the whole-cell samples), Cal (●●●●), and the Cal signal peptide (●●●●●●●●●●) are indicated. Numbers at left indicate molecular mass in kilodaltons.

RESULTS

Lipid modification and processing of pCal. Previous studies on the processing and lipid modification of Cal have demonstrated that these reactions take place very slowly compared to those of other lipoproteins and that the signal peptide is stable after cleavage from the precursor and accumulates in the inner membrane (3, 4, 13). Because it is difficult to unambiguously identify the various assembly intermediates of Cal in whole-cell samples (4), we used immunoprecipitation with the anti-Cal monoclonal antibody CA1 (14) in conjunction with pulse-chase analysis with [35S]methionine to study the processing and lipid modification reactions. W3110(pKAN) cells were induced for the production of colicin A and Cal with mitomycin C, pulsed for 1 min with [35S]methionine, and chased for 30 min with unlabeled methionine. Autoradiograms of SDS-PAGE gels of the samples showed the immediate appearance of precursor Cal (pCal) and its conversion, with a half-life of approximately 10 min, to mature, lipid-modified Cal, while lipid modified precursor Cal (pCalm) could not be identified, since it comigrated with the major lipoprotein in this gel system (Fig. 1). The bands corresponding to pCal, pCalm, and mature Cal could all be observed throughout the chase period in the CA1 immunoprecipitates, indicating that both the lipid modification of pCal and the cleavage of pCalm are limiting reactions in the processing of this precursor. To confirm the identities of the lipid-modified forms of Cal, they were immunoprecipitated from [3H]glycerol-labeled cells treated or not with globomycin, an antibiotic which inhibits processing by signal peptidase II and results in a pCalm accumulation (5, 9, 18). The autoradiograms of these immunoprecipitates indicated that only pCalm and mature Cal were labeled by the [3H]glycerol, and when the cells were treated with globomycin, only the band corresponding to pCalm could be immunoprecipitated (Fig. 2).

The autoradiogram in Fig. 1 also shows the accumulation in the whole-cell samples of a fourth band, with an apparent Mr of ~2,000, during the lipid modification and processing reactions. The absence of this band during inhibition of processing by either globomycin (5) or mutation (12) and the results of differential labeling studies (6) have demonstrated that it is the

FIG. 1. Lipid modification and processing of pCal. W3110 cells containing the colicin A plasmid pKAN were induced with mitomycin C or not induced, pulsed for 1 min with [35S]methionine, and chased with unlabeled methionine. After the periods of chase in minutes indicated at the top of each lane, whole-cell samples or Cal immunoprecipitates were separated by SDS-PAGE and visualized by autoradiography. The positions of migration of pCal (C), pCalm (●), Cal (●●●●), and the Cal signal peptide (●●●●●●●●●●) are indicated. Numbers at left indicate molecular mass in kilodaltons.

FIG. 2. Immunoprecipitation of pCalm and Cal after [2-3H]glycerol labeling in the presence and absence of globomycin. W3110 degP cells containing pKAN were left uninduced (lanes 1, 2, 5, and 6) or induced with mitomycin C (lanes 3, 4, 7, and 8), and aliquots of each culture were treated with 50 μg of globomycin per ml (lanes 1, 4, 5, and 8). The cells were then labeled with [2-3H]glycerol and either directly electrophoresed (lanes 1 to 4) or immunoprecipitated with CA1, with the immunoprecipitates then being electrophoresed (lanes 5 to 8). The positions of migration of pCalm (●), Cal (●●●●), and pLppm (●●●●●●●●●●) are indicated. Numbers at left indicate molecular mass in kilodaltons.
stable Cal signal peptide which is cleaved from pCal\(^{20}\) by signal peptidase \(\text{II}\) during the processing reaction.

**In vitro mutagenesis of cal.** We used site-directed mutagenesis, guided by previous studies of lipoprotein processing (10, 11), to determine why the Cal signal sequence is so slowly processed and degraded. Cal has the signal sequence Met Lys Lys Ile Ile Ile Cys Val Ile Leu Leu Ala Ile Met Leu Leu Ala Ala. Although the consensus lipoprotein cleavage sequence contains either Gly or Ala in the \(-1\) position (relative to the cleavage site), we created the mutation Ala\(_{18}\) → Gly\(_{18}\), because Gly is the most commonly occurring \(-1\) residue in lipoproteins and because Ala has a much higher propensity than Gly for \(\alpha\)-helical conformation (7), whereas a \(\beta\)-turn structure in this region has been suggested to be important for efficient processing of the major lipoprotein precursor (16). The pCal signal sequence also differs from most others in having no Ser or Thr residue at the end of the hydrophobic core, 5 to 7 residues amino-terminal to the cleavage site. The termination of a core hydrophobic \(\alpha\)-helix is also thought to be important to signal sequence structure and function (37, 38), and we therefore changed Ile\(_{13}\) to the more polar, turn-promoting Ser.

**Processing and degradation of the Gly\(_{18}\) and Ser\(_{13}\) Cal signal peptides.** The plasmids containing the cal mutations were transformed into W3110 cells and pulse-chased and immunoprecipitated as before. For each of the mutations, dramatic increases in the rate of pCal processing were observed, as shown in Fig. 3. The maturation of the Gly\(_{18}\) precursor was such that the majority of the labeled pCal had been processed within 1 min of the end of the pulse, while most of the labeled Ser\(_{13}\) derivative had been processed after 5 min. For both of the mutants, the increased processing rate was such that it obscured the precursor-product relationship between pCal and mature Cal that is evident during the processing of the wild-type protein (Fig. 1). The Ser\(_{13}\) mutation also had a dramatic effect on the degradation of the signal peptide itself (Fig. 3). In contrast to both wild type and the Gly\(_{18}\) mutant, the hydrolysis of the signal peptide of this mutant closely followed the maturation of the precursor. In some gels, including that shown in Fig. 3, this degradation appeared to proceed via a cleavage which gave rise to a subfragment slightly lower in molecular weight than the intact signal peptide, but this subfragment could not always be reliably observed.

**Processing of the Ser\(_{13}\)Gly\(_{18}\) double mutant.** Since both the Ser\(_{13}\) and Gly\(_{18}\) mutations had a strong positive effect on the processing of pCal, a plasmid which encoded a pCal which contained both of these amino acid changes was constructed. A comparison of its processing with that of the Gly\(_{18}\) pCal is shown in Fig. 4. This pCal derivative was processed so rapidly that a \([35\text{S}]\)methionine pulse of 15 s rather than 1 min was required for its analysis. The majority of Ser\(_{13}\)Gly\(_{18}\) pCal synthesized during the pulse was matured within the following 30 s, with no accumulation of the pCal\(^{29}\) intermediate, and the signal peptide was hydrolyzed with a half-life of less than 2 min. As found in the longer pulse-chase experiments, the Gly\(_{18}\) pCal, the more rapidly processed of the two single mutation derivatives, was essentially completely processed within 5 min of the end of the pulse and released a stable signal peptide. Little processing of wild-type pCal was observed in these short pulse-chase experiments (data not shown).

**Function of the mutant Cal proteins in colicin A release and quasilysis.** In order to assess the biological activities of Cal proteins which were much more rapidly lipid modified and processed than wild-type Cal or were synthesized with unstable signal peptides, we examined colicin A release and quasilysis in cells induced for these altered Cal proteins. In preliminary experiments in which the various mutants were grown in rich medium in preparation for induction with mitomycin C, we observed that the cells containing the Ser\(_{13}\) mutation and especially those containing the Ser\(_{13}\)Gly\(_{18}\) mutations grew more slowly than did the wild type or the Gly\(_{18}\) mutant. Inspection of the sequence of the mutant cal genes indicated that the creation of the Ser\(_{13}\) missense mutation had altered the –10 region of the colicin A immunity gene \(cai\) (which is adjacent to and transcribed in the opposite orientation from \(cai\) [21]) from CATGAT to CATGGA and was thus likely interfering with expression of the immunity protein. The Gly\(_{18}\) mutation also involves the substitution of a base in the \(cai\) promoter region, from –29G to C, and this may have further decreased the efficiency of the promoter, leading to the greater negative effect on the double mutant. Evidence in support of this interpretation was obtained when lawns of the wild-type and mutant strains were plated and spotted with various dilutions of a colicin A preparation, which demonstrated that the
The effect on processing observed with the Ser13 mutation, either alone or in combination with the Gly18 mutation, indicates that the processing enzymes also have a strong preference for a Cal signal peptide which contains Ser rather than Ile at this position, and when the Ser13 and Gly18 mutations were combined, the resulting pCal derivative was completely processed within seconds of its synthesis, as is observed for the precursors of most other secretory proteins. The Cal signal sequence is positively charged and contains a strongly hydrophobic central core, and thus, aside from the absence of a moderately polar residue at the end of the hydrophobic core and the presence of an Ala rather than a Gly in the −1 position, it strongly resembles that of the major lipoprotein in structure. It is therefore likely that the Ile13 and Ala18 residues of this peptide are the primary determinants of its poor quality as a substrate for the processing enzymes.

The striking change in the rate of degradation of the signal peptide when Ser replaced Ile at position 13 indicates that this region of the signal sequence is also critically important for the initial hydrolytic attack by the signal peptide peptidase. It is not yet clear whether it is the presence of the Ile residue itself or the conformation that it induces which is responsible for the stability of the wild-type signal peptide. Since the relative stability of the peptide changed in different ways can be monitored in these cells, it should be possible to specifically address these alternatives via the introduction of other mutations. The sequence specificities of two putative signal peptide peptidases, protease IV (25) and oligopeptidase A (36), suggest that neither would degrade the mutant Cal signal peptide more efficiently than the wild type. Although these enzymes have been shown to degrade signal peptides in vitro, the existence of mutants unable to produce them indicates that they are not necessary for signal peptide degradation in vivo, suggesting that multiple proteases may be involved in the process (25, 30).

The ability to alter the slow processing and signal sequence stability of the Cal protein has allowed us to examine the requirements for these features of Cal metabolism for its biological functions. The colicin secretion assays indicated that a more rapidly processed Cal, with or without a stable signal peptide, is at least as efficient as the wild-type protein in causing the release of the accumulated colicin and other proteins from the producing cells. In addition, all of these mutant Cal proteins were equally effective at causing quasiliyis of the culture, and as for the wild type, this was accompanied by a precipitous drop in cell viability. These results indicate that neither the slow processing nor the stability of the signal peptide is a requirement for these functions in vivo. In studies of the cloacin DF13 lysis protein, it was found that replacement of the entire signal sequence with the rapidly processed and degraded signal sequence of Braun’s lipoprotein resulted in a lysis protein that was much less efficient in cloacin release than the wild type, although quasiliyis and lethality were less affected. This suggested that the stable signal peptide is involved in the process of cloacin release, perhaps through the formation of a complex between the lysis protein and the signal peptide (23). It is also clear from the data of a number of studies that the overproduction of stable signal peptides can be lethal to E. coli and in some cases can also cause lysis of the cell (20, 33, 35). These findings do not, however, directly address the role of the stable signal peptides in the release of colicins that occurs in vivo when they are produced as a consequence of the processing of colicin lysis proteins. Our findings do not indicate whether the Cal signal peptide would be lethal to cells if it was produced in isolation, but they do demonstrate that mature, lipid-modified Cal, in the absence of either a stable signal peptide or processing intermediates, is fully capable of

FIG. 5. Quasiliyis of cells induced for wild-type and mutant Cal synthesis. Cultures were grown in Luria-Bertani medium and induced with mitomycin C at the point indicated by the arrow (open symbols) or not induced (closed symbols). The effects of the Cal mutations created here on the pro-

strains containing the Ser13 and the Ser13Gly18 mutations were much more sensitive to the colicin than was the wild type (data not shown). In order to circumvent the killing of the strains containing these mutations during the colicin secretion assays, we introduced the previously isolated BD2 deletion into the cau gene of each of the Cal mutant plasmids. The BD2 deletion removes amino acids 15 to 30 of colicin A and inactivates it, but has no effect on its secretion (1, 2). W3110 cells containing pBD2 plasmid derivatives with either wild-type cal or the cal signal sequence mutations grew normally, and when induced with mitomycin C, all underwent quasiliyis in a manner typical of cultures induced for colicin A synthesis, except that each of the mutants began quasiliyis slightly earlier than the wild type (Fig. 5). SDS-PAGE analysis of the cells and supernatants obtained by centrifugation of the cultures at various times after induction demonstrated that the wild type and each of the mutants was produced and released the BD2 derivative of colicin A with equal efficiency (data not shown). Finally, plate viability assays showed that induction of the mu-
	
	
	

in the rest of the cleavage region, which may include this region of the major lipoprotein signal sequence was examined by in vitro mutagenesis, the Gly→Ala mutation in the −1 position had a pronounced negative effect on the rate of processing only when combined with mutations in the +3 and +4 positions (16).
performing the biological function of lysis proteins—colicin release accompanied by quasilysis and cell death. This in turn indicates that the only function that the lysis protein signal peptide is required to perform in colicinogenesis is that for which it is named—to act as a signal which directs the lysis protein to the envelope.

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