Secretion of Recombinant Proteins via the Chaperone/Usher Pathway in *Escherichia coli*

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F1 antigen (CafI) of *Yersinia pestis* is assembled via the CafIM chaperone/CafIA usher pathway. We investigated the ability of this assembly system to facilitate secretion of full-length heterologous proteins fused to the CafI subunit in *Escherichia coli*. Despite correct processing of a chimeric protein composed of a modified CafI signal peptide, mature human interleukin-1 β (hIL-1 β), and mature CafI, the processed product (hIL-1 β :CafI) remained insoluble. Coexpression of this chimera with a functional CafIM chaperone led to the accumulation of soluble hIL-1 β :CafI in the periplasm. Soluble hIL-1 β :CafI reacted with monoclonal antibodies directed against structural epitopes of hIL-1 β . The results indicate that CafIM-induced release of hIL-1 β :CafI from the inner membrane promotes folding of the hIL-1 β domain. Similar results were obtained with the fusion of CafI to hIL-1 β receptor antagonist or to human granulocyte-macrophage colony-stimulating factor. Following coexpression of the hIL-1 β :CafI precursor with both the CafIM chaperone and CafIA outer membrane protein, hIL-1 β :CafI could be detected on the cell surface of *E. coli*. These results demonstrate for the first time the potential application of the chaperone/usher secretion pathway in the transport of subunits with large heterogeneous N-terminal fusions. This represents a novel means for the delivery of correctly folded heterologous proteins to the periplasm and cell surface as either polymers or cleavable monomeric domains.

The chaperone/usher protein-assisted assembly pathway is the major pathway of fimbria assembly in the family of gram-negative bacteria, *Enterobacteriaceae* (29). In contrast to the complex general secretory (type II) (14) and contact-mediated (type III) (18) pathways, the chaperone/usher export machinery involves only two specific proteins, a periplasmic chaperone and usher protein, for export across the outer membrane. The periplasmic chaperone ensures correct folding of structural subunits and transports the folded subunit to the outer membrane usher protein, which mediates surface localization, apparently by forming a large gated channel (29).

Secretion systems utilizing the chaperone/usher pathway can be divided into two families based on structural features of the chaperones and cell surface structures (9, 36). A prototype of the first family is the *pap* gene cluster encoding the PapD chaperone and PapC usher, which mediate assembly of the composite rigid Pap pili of *Escherichia coli* (29). PapD contains two domains, each with a β -barrel and an immunoglobulin (Ig)-like fold (8). The *caf* gene cluster that produces and assembles the capsular F1 (CafI) antigen of *Yersinia pestis* is the best-characterized representative of the second family (2, 6, 7, 12, 22, 37). The genes encode a 26.5-kDa periplasmic chaperone (CafIM) (7) and a 90.4-kDa outer membrane protein (CafIA) (12), which together can mediate the surface assembly of CafI antigen (6) in recombinant *E. coli* cells (2, 13). CafIM-like periplasmic chaperones are characterized by an extended

variable sequence between the proposed F1 and G1 β -strands, a disulfide bond connecting these two strands, and an accessory N-terminal sequence (2, 36, 37). Together, these three features may form an extension to the binding domain, which is important for chaperone function (2, 22, 37). In contrast to *pap*-like gene clusters, all members of *caf*-like gene clusters are involved in the assembly of structures with a simple composition and a less rigid structure (fibrillae or capsule-like morphology) (9, 36).

The crystal structures of the PapD-PapK chaperone-adapter subunit complex (28) and the type 1 pilin FimC-FimH chaperone-adhesin complex (3) have revealed that these pilin structural subunits also have immunoglobulin-like folds, except that the seventh β -strand is missing, leaving part of the hydrophobic core of the subunit exposed. Binding of the chaperone G1 β -strand to the C-terminal β -strand of the pilin within this hydrophobic groove completes the pilin immunoglobulin fold (3, 28). This donor strand complementation interaction between periplasmic chaperone and structural subunit appears to occur at the level of the inner membrane and appears to be required for correct folding prior to release of the subunit from the inner membrane (10). Mutagenesis studies have provided strong evidence that the CafIM chaperone uses a similar β -donor strand complementation mechanism to promote correct folding of CafI subunit at the inner membrane, although in this case the chaperone-subunit interaction is mediated by a particularly long, alternating hydrophobic extension to the chaperone G1 β -strand (2, 22).

When expressed cytosolically in *E. coli*, recombinant human interleukin-1 β (hIL-1 β) can be produced in a fully soluble and

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TABLE 1. Oligonucleotides used in this study

Oligonucleotide	Sequence
CAF-RI.....	5'-GGGAATTCAGAGGTAATATATGAAAAAATC-3'
IL-PST.....	5'-CCGCCTGCAGATGCGGCACCTGTACGATCACTG-3'
CAF-PST.....	5'-CCGCCTGCAGTTGCAATAGTTCCAAATA-3'
IL-Primer.....	5'-AGAACACCACTTGTGTGCTCC-3'
Blunt.....	5'-TGGAATCTATTGCAACTGCAAAATGCGGCACCTGTACGA-3'
3AA.....	5'-GCAACTGCAAAATGCGGCAGATTTAGCACCTGTACGATCACTG-3'
IL-BamHI.....	5'-ACCGGATCCACCTCCACCAGATCCACCTCCGGAAGACAAATTGTCATGG-3'
BamHI-Caf.....	5'-GGTGGATCCGGTGGTGGTGGATCTGCAGATTTAATGCAAGCAC-3'
Caf-SalI.....	5'-GCCAAGCTTGTTCGACGAGGGTTAGGCTCAAAGT-3'
SBEKP-1.....	5'-TCGACAGATCTCGAATTCGGTACCGGCTGCA-3'
SBEKP-2.....	3'-GTCTAGAGCTTAAGGCCATGGCCG-5'
STOP.....	5'-GATCATTAAATTAAT-3'
TRC.....	5'-CCAGATCTGGCAAAATATTCTGAAATG-3'
BLUNT-GM-CSF.....	5'-ATCGGAAATGTTTCGACCTTCAAG-3'
GM-CSF-Kpn2I.....	5'-ATTATTCGGACTCCTGCACTGGTTCCCAGC-3'
NcoI-IL-1ra.....	5'-GGAATCCATGGAGGGAAGAT-3'
IL-1ra-Kpn2I.....	5'-ATTATTCGGACTCCTGCTGAAAGTAG-3'
Kpn2I-IL-1ra.....	5'-ATGCGACCCTCCGGAAGAAAATCC-3'
KpnI-Caf1M.....	5'-GTTGTCTGGTACCATTCCGTAAGGAGG-3'
Caf1M- <i>Alw44I</i>	5'-GTTAACGTGCACACAGGAACAGC-3'
O1.....	5'-CATCGCAACTGCTAACGCAGCAGACGATCCCT-3'
O2.....	5'-CCGAGGGATCGTCTGCTGCGTTAGCAGTTGCGATGGTAC-3'

active conformation and can be released by osmotic shock (11, 34). hIL-1 β can also be directed to the Sec secretion pathway by fusion to a signal peptide (4, 5). However, despite the fact that the signal peptide was cleaved when hIL-1 β was targeted by this route, no soluble hIL-1 β was released into the periplasm. The processed form appeared to be incapable of correctly folding and formed membrane-associated aggregates. Similar results were obtained with the closely related hIL-1 receptor antagonist (hIL-1ra) (reference 31 and our unpublished results). Periplasmic localization of human granulocyte-macrophage colony-stimulating factor (hGM-CSF) fused to the signal peptide of OmpA (17) or of Caf1 (27) has been more successful, although the majority of the processed protein was still recovered with insoluble cell debris.

As the Caf1M chaperone apparently aids periplasmic folding and prevents aggregation of newly translocated Caf1 subunit (2, 37), the ability of this system to enhance solubilization of recombinant eucaryotic proteins was investigated using the cytokines, hIL-1 β , hIL-1ra, and hGM-CSF. In this system, genes encoding chimeric proteins were created in which the cytokine was sandwiched between the Caf1 signal peptide and the mature Caf1 subunit, leaving the C terminus of the Caf1 subunit free to interact with the chaperone. It is shown that regardless of the nature of the N-terminal heterologous protein, the Caf1 domain of the chimera remained free to interact with Caf1M and that this interaction enhanced the solubility of the periplasmic cytokine. Surface adhesins have frequently been investigated as carriers of short heterologous epitopes inserted within permissive sites of pilin subunits (15, 25, 30, 33). This study also provides the first evidence for localization of entire proteins to the cell surface of gram-negative bacteria using such an assembly system.

MATERIALS AND METHODS

Plasmids, bacterial strains, and culture conditions. Plasmids pKM4 (13), pPR-TGATG-hIL-1 β -tsr (24), pUC19-IL1ra (16), pFS2 (6), and pFMA1 (2) were used as a source of the genes *caf1*, *hIL-1 β* , *hIL-1ra*, *caf1M*, and *caf1A*,

respectively. Plasmid pFGM13 carrying the gene encoding a chimera of the Caf1 signal peptide with hGM-CSF under the *lac* promoter has been described (27). *E. coli* JM105 and NM522 (Stratagene) and JCB570 (*dsbA::kan*), kindly provided by J. Bardwell (University of Michigan, Ann Arbor, Mich.), were used as host strains. Bacteria were grown in M9 salts medium supplemented with 0.5% Casamino Acids (Difco) or Luria-Bertani medium (23) containing ampicillin (70 μ g/ml) and/or chloramphenicol (35 μ g/ml).

General DNA techniques. DNA manipulations and transformation of *E. coli* cells were performed as described by Maniatis et al. (23). Restriction enzymes, mung bean nuclease, and T4 DNA ligase were purchased from Promega. *Pfu* DNA polymerase (Stratagene) was used for PCR. Nucleotide sequencing was carried out using the TaqTrack sequencing kit (Promega). Oligonucleotides (Table 1) were from MedProbe.

Construction of pKKmod⁺Caf1-hIL-1 β , pKKmod⁺Caf1(-2)hIL-1 β , and pKKmod⁺Caf1(+3)hIL-1 β . The *EcoRI*-*PstI* fragment (about 110 bp) encoding the Caf1 5'-untranslated region and N-terminal part of the Caf1 signal peptide with the mutation Asn(-2)→Asp was generated by PCR with the CAF-RI and CAF-PST primers using pKM4 as a template, followed by *EcoRI* and *PstI* digestion of the PCR product. The *PstI*-*HindIII* fragment (about 60 bp) encoding the C-terminal part of Caf1 signal peptide joined to the N-terminal end of hIL-1 β was obtained by PCR using IL-PST and IL-Primer primers and pPR-TGATG-hIL-1 β -tsr as template, followed by digestion of the PCR product with *PstI* and *HindIII*. These two fragments were ligated together with the pUC19/*EcoRI*-*HindIII* vector fragment. The *EcoRI*-*HindIII* fragment from the resulting plasmid and the *HindIII*-*BamHI* fragment isolated from pPR-TGATG-hIL-1 β -tsr were ligated together with the *EcoRI*-*BamHI*-digested pUC19/*HindIII* vector (pUC19 with the *HindIII* site filled in and blunt-end ligated) to form psCaf1(-2)hIL-1 β . The point mutation G to A converting the *scf1*(-2)*hil-1 β* gene into the *scf1*-*hil-1 β* gene was made by a two-step PCR procedure using psCaf1(-2)hIL-1 β as template. In the first step, an intermediate PCR product was obtained with the mutagenic BLUNT primers and the M13 Sequence Primer (Promega). The intermediate PCR product was used as a primer for the second PCR step together with IL-Primer. The resulting PCR product was digested with *EcoRI* and *HindIII* and then ligated into corresponding sites of psCaf1(-2)hIL-1 β to form psCaf1-hIL-1 β . The *scf1*(+3)*hil-1 β* gene [psCaf1(+3)hIL-1 β] was constructed in a similar way using the mutagenic 3AA primer and psCaf1-hIL-1 β as template. DNA sequences of the *EcoRI*-*HindIII* fragments of all three hybrid genes were confirmed. To obtain expression plasmids, the *EcoRI*-*BamHI* fragments coding for the chimeric proteins were transferred into pKKmod, resulting in pKKmod⁺Caf1(-2)hIL-1 β , pKKmod⁺Caf1-hIL-1 β , and pKKmod⁺Caf1(+3)hIL-1 β (Fig. 1A).

Construction of expression-secretion vectors encoding hIL-1 β :Caf1, hGM-CSF:Caf1, and hIL-1ra:Caf1. The hIL-1 β part of hIL-1 β :Caf1 precursor was obtained by PCR using the IL-PST and IL-BamHI primers and psCaf1(-2)hIL-1 β as template. The Caf1 part of the hIL-1 β :Caf1 precursor was obtained by

PCR using the *Bam*HI-Cafl and Cafl-*Sal*I primers and pKM4 as a template. The PCR products were digested with restriction enzymes *Pst*I-*Bam*HI or *Sal*I-*Bam*HI, as appropriate, followed by triple ligation with the *Pst*I-*Sal*I vector fragment of pCafl(-2)hIL-1 β . To produce pCIC (Fig. 1B), an *Eco*RI-*Sal*I fragment was excised from the resulting plasmid and ligated into the *Eco*RI-*Sal*I vector fragment of pTrc99 Δ NcoI (pTrc99a [Pharmacia] with the *Nco*I site removed by mung bean nuclease and ligation). To create pCGC (Fig. 1B), the *gm-csf* gene was amplified from pFGM13 with primers BLUNT-GM-CSF and GM-CSF-Kpn2I to introduce a *Kpn*2I site at the 3' terminus. After treatment with *Kpn*2I, the fragment was ligated into the pFGM13 *Eco*RV-*Sal*I large fragment together with a *Kpn*2I-*Sal*I fragment from pCIC containing the Cafl coding region and spacer (Gly,Ser)₃ to produce pCGC. To create pCIRAC (Fig. 1B), the *il-Ira* gene was amplified from pUC19-IL-1ra using the Kpn2I-IL-1ra and M13 Sequence Primer primers, with concomitant introduction of a *Kpn*2I site at the 5' terminus of the gene via a silent mutation. To produce pFRA75, the resulting fragment was cut with *Kpn*2I and *Eco*RI and ligated with the *Kpn*I-*Eco*RI vector fragment of pFGM13 together with the O1 and O2 oligonucleotides to restore the common frame between the *scaf*I and *hil-Ira* genes. The *Kpn*2I site at the 3' terminus of the *hil-Ira* gene was introduced by PCR of pFRA75 with primers NcoI-IL-1ra and IL-1ra-Kpn2I. The amplified fragment was cut with *Nco*I and *Kpn*2I and ligated with the pCGC *Hind*III-*Kpn*2I large fragment together with the *Hind*III-*Nco*I fragment from pFRA75 to produce pCIRAC.

Construction of pACYC-based CafIM and CafIM-CafIA secretion vectors. pACYC-trx plasmid was created by cloning the small *Bam*HI-*Sca*I fragment from the pKK-trx plasmid (1) into pACYC184 (Pharmacia). The gene encoding CafIM was amplified from pFS2 by using primers KpnI-CafIM and CafIM-ApaLI. The PCR fragment was treated with *Kpn*I and *Apa*LI and cloned into the pACYC-trx *Kpn*I-*Apa*LI large fragment to produce pCafIM (Fig. 1C), which

carries the *cafIM* gene under the *tac* promoter. To produce pCaf1MA (Fig. 1C), the *ApaLI*-*ApaLI* fragment containing *cafIM* and *cafIA* genes under the *trc* promoter was excised from pFMA (2) and ligated into *ApaLI*-digested pCaf1M.

Construction of expression-secretion vectors where *caf1M*, *caf1A*, and *hIL-1 β* :*Caf1* precursor genes form an operon. These constructions, as shown in Fig. 1C, were based on pFMA1 (3), where genes for Caf1M, Caf1A, and Caf1 are under control of the *trc* promoter. To replace the Caf1 gene with an SBEKP synthetic polylinker, pMA-link was obtained by triple ligation of a pFMA1/*Pst*I-*Spe*I vector, a *Spe*I-*Pst*I fragment of pFMA1, and SBEKP-1 and SBEKP-2 oligonucleotides annealed together. pM-link was obtained from pMA-link by excision of a *Sal*I-*Sal*I fragment encoding Caf1A followed by self ligation of the vector. pA-link was obtained from pMA-link by excision of a *Bam*HI-*Bam*HI fragment encoding the C-terminal part of Caf1M. To interrupt the Caf1M translation frame, a stop codon was inserted by ligation of a self-complementary STOP oligonucleotide into the *Bam*HI site, resulting in loss of the *Bam*HI site. A fragment encoding the hIL-1 β :Caf1 precursor was excised from pCIC with *Eco*RI and *Sal*I, cloned into pBCSK⁺ (Stratagene), and recovered with *Eco*RI and *Kpn*I. To obtain pMA-CIC, pM-CIC, and pA-CIC, the *Eco*RI-*Kpn*I fragment was cloned into corresponding sites of pMA-link, pM-link, and pA-link, respectively. To create pM-Pr-CIC, DNA of the *trc* promoter and the 5' region of the hIL-1 β :Caf1 precursor gene was amplified by PCR using the TRC and CAF-Pst primers and pCIC as a template. The PCR product was digested with *Bgl*II and *Eco*RI and ligated into the corresponding sites of pM-CIC, pMA-Pr-CIC and pA-Pr-CIC were obtained by ligation of the *Bgl*II-*Kpn*I fragment from pM-Pr-CIC into corresponding sites of pMA-link and pA-link.

Induction and isolation of subcellular fractions. *E. coli* cells were grown to an absorbance at 600 nm of 0.5. For induction of protein expression, isopropyl- β -D-thiogalactopyranoside (IPTG; Sigma) was routinely added to maintain a final concentration of 0.5 mM and cells were grown for a further 1.5 to 2 h. Cells were

TABLE 2. Sequences of the last 6 residues of signal sequence and the first 10 residues of mature protein of the constructs used in this study^a

Amino acid sequence	Construct
-6-5-4-3-2-1 1 2 3 4 5 6 7 8 9 10	
... <u>I A T A N A</u> ↓A P V R S L N C T L	³⁵ Caf1-hIL-1β
... <u>I A T A N A</u> ↓A P V R S L N C T L	³⁵ Caf1(-2)-hIL-1β, hIL-1β:Caf1 precursor
... <u>I A T A N A</u> ↓A P V R S L N	³⁵ Caf1(+3)hIL-1β
... <u>I A T A N A</u> ↓A D D P S G R K S S	hIL-1ra:Caf1 precursor
... <u>I A T A N A</u> ↓A R P S G R K S S	Mature hIL-1ra
... <u>I A T A N A</u> ↓A D R S P S P S T Q	hGM-CSF:Caf1 precursor
... <u>I A T A N A</u> ↓A A P A R S P S P S T Q	Mature hGM-CSF

^a The sequence of the Caf1 signal peptide and three amino acids of mature Caf1 [in ³⁵Caf1(+3)hIL-1β] are underlined. ↓, proposed processing site. Charged amino acids are in bold. Amino acids formed as a result of mutagenesis to optimize export and processing are in italics. The first few amino acids of mature hIL-1ra and hGM-CSF are shown for comparison.

recovered by centrifugation. Cells were lysed by sonication with a Labsonic U Generator (B. Braun Diessel Biotech) and centrifuged at 16,000 × *g* for 20 min to recover soluble and pelleted proteins. Periplasmic proteins were recovered by osmotic shock extraction as previously described (37). The activity of the cytoplasmic enzyme glucose-6-phosphate dehydrogenase was monitored to control the purity of the periplasmic fraction (26). Following extraction of the periplasmic fraction, cells were suspended in 50 mM H₃PO₄-Tris (pH 6.8), sonicated, and centrifuged as described above to recover pelleted proteins. Pelleted proteins (some membranes plus inclusion bodies or aggregates) were extracted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2% SDS and 5% β-mercaptoethanol, heated at 100°C for 15 min, and subjected to SDS-PAGE.

Electrophoresis and IEF. Proteins were separated by 10 to 15% (wt/vol) PAGE in the presence of 0.1% SDS or by isoelectric focusing (IEF) using precast pI 3 to 9 gels on a Phast gel system (Pharmacia) and stained with Coomassie blue R-350.

Immunoblotting and ELISA. After SDS-PAGE or IEF, proteins were electrophoretically transferred to Hybond-C membrane (Amersham). Immunodetection of hIL-1β, Caf1-, and hGM-CSF-containing chimeric proteins, Caf1M, and Caf1A was performed using polyclonal rabbit anti-hIL-1β (Calbiochem), monospecific anti-Caf1, rabbit polyclonal anti-hGM-CSF (obtained and purified by T. Chernovskaya [2]), rabbit polyclonal anti-Caf1M (22), and polyclonal anti-Caf1A (raised in mice against SDS-PAGE-purified Caf1A) antibodies, respectively. Binding of the primary antibodies was visualized by peroxidase-labeled anti-rabbit (Calbiochem) and anti-mouse (Amersham) antibodies using an ECL kit (Amersham). Enzyme-linked immunosorbent assay (ELISA) was performed as described previously (2). In addition to antibodies used in Western blotting, monoclonal mouse antibodies to structural epitopes of hIL-1β from clones 6E10 and 11E5 (HyTest) were used in ELISA for the detection of hIL-1β-containing chimeric proteins.

Protein sequencing. After partial purification of periplasmic fractions by chromatography on a DEAE-Sepharose CL-6B (Pharmacia) column (0 to 500 mM NaCl gradient in 50 mM Tris-HCl buffer at pH 7.5), proteins were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Amersham). The desired bands were excised and placed onto a polybrene-coated and precycled glass fiber filter. Amino acid sequence analyses were performed with an Applied Biosystems model 477A protein sequencer equipped with on-line Applied Biosystems model 120A phenylthiohydantoin amino acid analyzer.

Trypsin digestion of permeabilized cells. Induced cells were permeabilized with sucrose-EDTA and treated for 1.5 h with 0.5 mg of trypsin/ml as previously described (19).

Detection of surface-assembled antigens. For immunofluorescence quantitation, cells from induced cultures were incubated sequentially with a 1:500 dilution of anti-Caf1 antibody or a 1:100 dilution of anti-hIL-1β serum and a 1:50 dilution of anti-rabbit immunoglobulin G-fluorescein conjugate (Sigma). Fluorescence was measured with a Victor (Wallac) plate reader. Cell agglutination experiments were made using a reticulocyte monoclonal diagnostic kit for the detection of *Y. pestis* (Middle Asian Research Institute, Alma-Ata, Kazakhstan).

RESULTS

Optimization of the Caf1 signal peptide: hIL-1β fusion for secretion across plasma membrane. The presence of a net positive charge at the N terminus of a mature protein often

disturbs plasma membrane translocation and the processing of precursor polypeptides in *E. coli*. This can be alleviated by reducing the net positive charge or optimizing the signal peptide (20, 21). Hence, prior to testing the ability of the Caf system to solubilize recombinant hIL-1β, different variants encoding the Caf1 signal peptide fused to hIL-1β were created to test for the compensation of the Arg residue at position +4 of mature hIL-1β (Table 2). pKKmod³⁵Caf1-hIL-1β encoded the Caf1 signal peptide joined directly to the first amino acid of hIL-1β. pKKmod³⁵Caf1(-2)-hIL-1β encoded the same chimera, but with an Asn(-2)→Asp mutation in the Caf1 signal peptide, and pKKmod³⁵Caf1(+3)hIL-1β encoded a fusion containing an additional three N-terminal amino acids of mature Caf1 to preserve the natural processing site of Caf1 precursor (Fig. 1A; Table 2). Expression from either pKKmod³⁵Caf1(-2)-hIL-1β or pKKmod³⁵Caf1(+3)hIL-1β led to the production of bands corresponding to precursor and mature hIL-1β (Fig. 2, lanes 8 and 9), whereas expression of pKKmod³⁵Caf1-hIL-1β

FIG. 2. Expression of ³⁵Caf1-hIL-1β, ³⁵Caf1(-2)hIL-1β, and ³⁵Caf1(+3)hIL-1β. Coomassie blue-stained SDS-PAGE gel of soluble (lanes 2 to 5) and insoluble (pellet) (lanes 6 to 9) proteins, obtained following sonication, from *E. coli* JM105 cells transformed with pKKmod (lanes 2 and 6), pKKmod³⁵Caf1-hIL-1β (lanes 3 and 7), pKKmod³⁵Caf1(-2)hIL-1β (lanes 4 and 8), and pKKmod³⁵Caf1(+3)hIL-1β (lanes 5 and 9). hIL-1β was loaded as a control (lanes 1 and 10). The arrow indicates the position of mature hIL-1β identified by N-terminal sequencing. Processed and unprocessed ³⁵Caf1(+3)hIL-1β migrated with a slightly slower electrophoretic mobility due to the three-amino-acid insert.

resulted in only one additional protein with a molecular weight corresponding to that of the unprocessed precursor (Fig. 2, lane 7). In contrast to the absence of the processing of Caf1-hIL-1 β , approximately 40% of both $^{\text{s}}\text{Caf1}(-2)\text{hIL-1}\beta$ and $^{\text{s}}\text{Caf1}(+3)\text{hIL-1}\beta$ were processed. However, the processed products were not secreted into the periplasm and evidently remained in an insoluble form (Fig. 2, lanes 6 to 9). Negligible amounts were recovered from the soluble fraction (Fig. 2, lanes 2 to 5). The low centrifugal force ($16,000 \times g$ for 20 min) by which the processed hIL-1 β was almost completely recovered from the sonicated cells was consistent with aggregate formation of the processed cytokine at the inner membrane or in the periplasm. Since $^{\text{s}}\text{Caf1}(-2)\text{hIL-1}\beta$ forms an intact mature hIL-1 β after signal peptidase processing, it was chosen for further investigations.

The processing of ³⁵S-Caf1(-2)hIL-1β was significantly less efficient in rich Luria-Bertani medium than in poor M9 medium (data not shown). Alteration in the growth temperature and concentration of IPTG inducer increased the rate of expression of precursor but did not lead to any significant increase in the final amount of processed chimeric protein (data not shown). Also, there was no increase in the level of soluble

periplasmic hIL-1 β recovered with any of the variations in growth conditions tested.

Secretion of a hIL-1 β :Caf1 chimeric protein across the plasma membrane. To probe the cellular localization of the insoluble processed $^{\text{s}}$ Caf1(-2)hIL-1 β , we performed trypsin digestion of permeabilized cells. In this procedure, trypsin penetrates the periplasm of cells and digests soluble proteins as well as membrane-bound proteins exposed to the liquid phase. In contrast to the $^{\text{s}}$ Caf1(-2)hIL-1 β precursor, almost all of the processed $^{\text{s}}$ Caf1(-2)hIL-1 β was digested by trypsin (Fig. 3A, compare lanes 5 and 8). The result corroborates that processed, insoluble $^{\text{s}}$ Caf1(-2)hIL-1 β was at least partially translocated across the inner membrane and accessible to the liquid phase of the periplasm. This construct therefore represented a good experimental model to test the ability of the Caf1M chaperone to promote solubilization of problem recombinant proteins in the periplasm.

To create a binding site for Caf1M, pCIC, which encodes ^sCaf1(−2)hIL-1β linked via a (Gly₄Ser)₃ spacer to mature Caf1 (Fig. 1B), was constructed. *E. coli* JM105 cells expressing this construct produced a chimeric protein that was apparently even more efficiently processed than ^sCaf1(−2)hIL-1β (Fig.

3A, lanes 5 and 6). Precision of the signal peptidase processing step was confirmed by sequencing the N terminus of mature hIL-1 β :Caf1 (Table 2). However, as was the case with 3 Caf1(-2)hIL-1 β (Fig. 3A, lanes 2 and 5), only a minor fraction of mature hIL-1 β :Caf1 could be extracted by osmotic shock. Not surprisingly, the major fraction remained associated with the shocked cells (pellet fraction) (Fig. 3A, lane 6). Although hIL-1 β :Caf1 was more resistant to trypsin, it was still partly accessible to the protease and appeared to be mainly digested at the C terminus of the chimera (Caf1 domain)(Fig. 3A, lane 9).

Caf1M chaperone-enhanced solubilization of the hIL-1 β :Caf1 chimera. To assess the influence of Caf1M on the solubility of the hIL-1 β :Caf1 chimera, hIL-1 β :Caf1 precursor and Caf1M were coexpressed in *E. coli* NM522 cells from pM-Pr-CIC. Following a 1.5-h induction with IPTG, a dramatic (10- to 20-fold) increase in the recovery of periplasmic hIL-1 β :Caf1 was evident (Fig. 3A, lanes 3 and 4, and Fig. 3B, lanes 1 and 4). Cells expressing Caf1M together with hIL-1 β :Caf1 precursor were more viable than cells expressing only hIL-1 β :Caf1 precursor or Caf1M. This observation is consistent with Caf1M enhancing folding and preventing formation of toxic hIL-1 β :Caf1 aggregates. Caf1M was unable to facilitate periplasmic secretion of processed 3 Caf1(-2)hIL-1 β or of an hIL-1 β :Caf1 precursor mutant with a frameshift in the DNA encoding the (Gly₄Ser)₃ spacer. This demonstrates that specific binding of Caf1M to the Caf1 part of the fusion was critical for the observed promotion of hIL-1 β :Caf1 solubilization. In the presence of the outer membrane protein, Caf1A, there was possibly a small decrease in periplasmic chimera (Fig. 3B).

Interaction of Caf1M with hIL-1 β :Caf1 was examined directly by IEF of periplasmic extracts. Three major bands (pI 8.7, 8.2, and 5.9) which stained with Coomassie blue following IEF of the periplasmic extract from NM522 cells carrying plasmid pM-CIC (Fig. 4A, lane 2) also reacted with anti-Caf1M antibody (Fig. 4B, lanes 1 and 2). Two of these bands (pI 8.7 and 8.2) were also detected following IEF and immunoblotting of a periplasmic extract of cells expressing Caf1M alone and represented free dimeric and monomeric Caf1M (Fig. 4B, lane 4). Only the third band, which had an isoelectric point of 5.9 and which reacted with anti-IL-1 β antibody (Fig. 4C, lanes 1 and 2), represented the hIL-1 β :Caf1-Caf1M complex. An additional ladder of bands at pI 5.2 was clearly visualized with anti-IL-1 β antibody. The same ladder of bands was also detected with anti-Caf1M antibody following longer exposure of film to the immunoblot of Fig. 4B (data not shown). In the absence of the Caf1A outer membrane protein, a functional Caf1M chaperone leads to the formation of periplasmic polymers of Caf1 subunit (22). Such periplasmic polymers, which exhibit the same characteristic IEF banding pattern at pI 5.2, have been purified and identified as Caf1M-[Caf1]_n complexes (A. V. Zavialov, unpublished results). Hence, the ladder of bands observed in this study (Fig. 4C, lanes 1 and 2) would appear to represent polymers of hIL-1 β :Caf1 of increasing size capped by Caf1M.

In the absence of Caf1M, there was significant degradation of the hIL-1 β :Caf1 chimera. This was observed in pulse-chase experiments (not shown) and in immunoblottings of periplasmic fractions. Since the 19- to 22-kDa degradation intermediate detected in periplasmic fractions (Fig. 3B, lanes 1 and 5)

FIG. 4. IEF identified Caf1M chaperone hIL-1 β :Caf1 complex. (A) IEF gel stained with Coomassie blue. pI marker proteins and periplasmic extract of NM522 cells carrying the pM-CIC plasmid were loaded on lanes 1 and 2, respectively. The arrow shows the position of the major hIL-1 β :Caf1-Caf1M complex. (B) Immunoblotting of an IEF gel of periplasmic extracts from NM522 cells carrying plasmid pM-CIC (lanes 1 and 2), pTrc99a vector (lane 3), and pTCA (2) (Caf1M only) (lane 4) analyzed with anti-Caf1M antibody. Bands at pI 8.7 and 8.2 were identified as Caf1M monomer and dimer, respectively, by IEF of purified proteins (not shown). (C) Immunoblotting from IEF gel of the same samples shown in panel B with anti-IL-1 β antibody. Arrows show polymeric forms of hIL-1 β :Caf1 capped with Caf1M. The pI of the complex increased as the amount of hIL-1 β :Caf1 subunits in polymer increased.

reacted with anti-hIL-1 β antibody but not with anti-Caf1 antibody, the Caf1 part of hIL-1 β :Caf1 appeared to be degraded more rapidly than the IL-1 β domain (data not shown). In the presence of Caf1M, hIL-1 β :Caf1 was stable (Fig. 3B, lanes 3 and 4). Most importantly, periplasmic hIL-1 β :Caf1 expressed in the presence of Caf1M reacted well with monoclonal antibodies to structural epitopes of hIL-1 β in an ELISA. Periplasmic extracts of *E. coli* JM105 cells coexpressing hIL-1 β :Caf1 precursor and Caf1M (from pCIC and pCaf1M) displayed on average a 14-fold-stronger signal with anti-IL-1 β monoclonal antibodies, clone 6E10, and a 16-fold-stronger signal with anti-IL-1 β monoclonal antibodies, clone 11E5 (HyTest, Turku, Finland), than periplasmic extracts of *E. coli* JM105 cells expressing hIL-1 β :Caf1 precursor alone (pCIC). As these monoclonal antibodies did not react with denatured hIL-1 β :Caf1 in a Western blot assay, this provides some evidence that the hIL-1 β part of the hIL-1 β :Caf1 chimera was correctly folded when secreted in the presence of Caf1M.

Caf1M promotes solubilization of hGM-CSF:Caf1 and hIL-1 α :Caf1 chimeras. Two other chimeras were made to test the general ability of Caf1M to promote the solubilization of secreted proteins in *E. coli*: (i) the hGM-CSF:Caf1 precursor consisting of 3 Caf1 signal sequence, growth factor hGM-CSF with mutations Pro2Ala3 $\rightarrow$ Asp, spacer Ser(Gly₄Ser)₃, and mature Caf1, and (ii) the hIL-1 α :Caf1 precursor consisting of a

FIG. 5. Caf1M facilitates secretion of hIL-1ra:Caf1 and hGM-CSF:Caf1. Immunoblottings of periplasmic samples from *E. coli* JCB570 cells transformed with pCGC (lane 1), pCGC and pCaf1M (lane 2), pCIRAC (lane 3), and pCIRAC and pCaf1M (lane 4) were performed using anti-Caf1 polyclonal antibodies. Protein expression was induced with 0.5 mM IPTG for 1.5 h.

^sCaf1 signal sequence, mature hIL-1ra with mutation Arg1 to AlaAspAsp, spacer Ser(Gly₄Ser)₃, and mature Caf1. As before, acidic residues were introduced close to the processing site to neutralize the N-terminal positive charge (Table 2) and optimize precursor export and processing. The expression of the resulting precursors in *E. coli* JCB570 cells from the plasmids pGCG and pCIRAC led to the accumulation of the mature proteins hGM-CSF:Caf1 and hIL-1ra:Caf1, respectively, in the pellet fractions (data not shown). As with hIL-1 β :Caf1 alone, only a minor fraction of hGM-CSF:Caf1 and hIL-1ra:Caf1 was detected in the periplasm (Fig. 5, lanes 1 and

3). However, when *E. coli* JCB570 cells were cotransformed with pCGC and pCaf1M or pCIRAC and pCaf1M, the levels of periplasmic hGM-CSF:Caf1 and hIL-1ra:Caf1 increased about 10-fold (Fig. 5, lanes 2 and 4).

Assembly of IL-1 β :Caf1 chimera on the cell surface. As the Caf1M chaperone could promote formation of soluble hIL-1 β :Caf1-Caf1M complex in the periplasm, the ability of the complete Caf system (Caf1A outer membrane usher together with Caf1M) to mediate surface localization of hIL-1 β :Caf1 was investigated. hIL-1 β :Caf1 precursor was coexpressed with Caf1M and Caf1A in *E. coli* NM522 cells harboring either pMA-CIC or pMA-Pr-CIC. Surface-exposed hIL-1 β :Caf1 could be detected in these strains in a hemagglutination assay using reticulocytes sensitized with monoclonal anti-Caf1 antibody (Table 3). hIL-1 β :Caf1 was also detected on the surface of *E. coli* cells by quantitative immunofluorescence using either polyclonal anti-Caf1 or polyclonal anti-IL-1 β antibody (Table 3). Cells expressing only hIL-1 β :Caf1 (pCIC) or hIL-1 β :Caf1 together with Caf1M (pM-CIC or pM-Pr-CIC) were negative in both assays. The amount of surface immunodetected hIL-1 β :Caf1, however, was about 10-fold less than the amount of wild-type Caf1 antigen detected on the surface of *E. coli* NM522 harboring pFMA1 (Table 3).

DISCUSSION

This study elucidates a novel approach for heterologous expression of problem recombinant proteins in the periplasm of *E. coli*. Interaction between the Caf1M molecular chaperone and the Caf1 structural subunit at the periplasmic surface of the plasma membrane was used successfully to promote the solubilization of otherwise insoluble recombinant cytokines in the periplasm. This was achieved by creating genes encoding chimeric proteins in which the cytokine was sandwiched between the Caf1 single peptide and the mature Caf1 subunit, leaving the C terminus of the Caf1 subunit free to interact with the chaperone. Three different cytokines were tested, of which two had primarily a β -structure (hIL-1 β and hIL-1ra [35]) while the third was an α -helical protein (hGM-CSF) (32). Regardless of the nature of the N-terminal heterologous protein, the Caf1 domain of the chimeric protein remained free to

TABLE 3. Detection of surface-exposed hIL-1 β :Caf1^a

Plasmid	Hemagglutination assay results		Immunofluorescence assay results			
	Effect	Concn (cells/ml)	Anti-Caf1 antibody		Anti-IL-1 β antibody	
			Effect	Counts	Effect	Counts
pCIC	—	Up to 10 ⁹	—	345 $\pm$ 185	—	255 $\pm$ 65
pM-CIC	—	Up to 10 ⁹	—	240 $\pm$ 140	—	180 $\pm$ 30
pMA-CIC	+	1.2 $\times$ 10 ⁷	+	3,215 $\pm$ 475	+	1,665 $\pm$ 325
pM-Pr-CIC	—	Up to 10 ⁹	—	305 $\pm$ 65	—	250 $\pm$ 70
pMA-Pr-CIC	+	3.9 $\times$ 10 ⁶	+	4,830 $\pm$ 820	+	2,560 $\pm$ 450
pFMA1	+	4.9 $\times$ 10 ⁵	+	38,440 $\pm$ 5,350	—	75 $\pm$ 45
pTrc99a	—	Up to 10 ⁹	—	145 $\pm$ 35	—	120 $\pm$ 10

^a NM522 cells carrying the indicated plasmids were incubated with 0.1 mM IPTG for 1 h. A hemagglutination assay was performed using goat reticulocytes sensitized with monoclonal anti-Caf1 antibody. The concentrations of cells at which 50% hemagglutination was observed (+) are shown. For the immunofluorescence assay, cells were incubated sequentially with anti-Caf1 antibody or anti-IL-1 β antibody, and IgG-fluorescein conjugate and fluorescence were quantitated with a fluorescence plate reader. The mean background (1,430 counts) was subtracted. The presence of surface-exposed antigen (+) was judged by comparison with a negative control. Cells carrying plasmid pFMA1 assembling Caf1 antigen served as a positive control. Cells carrying plasmid pTrc99a served as a negative control.

interact with Caf1M, and this interaction enhanced the solubility of the periplasmic recombinant cytokine 10- to 20-fold.

hIL-1 β was selected for this study as it had previously been shown to form plasma membrane-associated aggregates when targeted to the periplasm of *E. coli* using the OmpA signal peptide (4, 5). Similar results were obtained with the Caf1 signal peptide in this study. Although a significant amount of 35 S-Caf1(-2)hIL-1 β was precisely processed, the mature protein was not secreted into the periplasm in a soluble form. Following sonication of cells expressing 35 S-Caf1(-2)hIL-1 β , the processed form was fully recovered in the pellet at a relatively low centrifugal force ($16,000 \times g$ for 20 min). It could also be completely degraded by trypsin in cells with a permeabilized outer membrane. These data are consistent with the translocation of hIL-1 β across the plasma membrane followed by aggregation of misfolded cytokine at the periplasmic surface of the plasma membrane. Not surprisingly, fusion of 35 S-Caf1(-2)hIL-1 β with mature Caf1 at the C terminus (hIL-1 β :Caf1 precursor) did not improve the recovery of soluble cytokine in the absence of Caf1M. Caf1 itself requires interaction with the Caf1M chaperone for correct folding and prevention of aggregate formation at the inner membrane (2, 22, 37). Indeed, trypsin digestion studies indicated that hIL-1 β :Caf1 may be more intimately associated with the inner membrane than mature 35 S-Caf1(-2)hIL-1 β , as a protected hIL-1 β fragment was recovered in trypsin-treated plasmolyzed cells. Tighter association with the inner membrane may be important in preventing misfolding of the hIL-1 β domain prior to interaction with Caf1M.

Coexpression of the hIL-1 β :Caf1 precursor with Caf1M resulted in a dramatic (10- to 20-fold) recovery of hIL-1 β :Caf1 in the soluble periplasmic fraction, with a corresponding increase in hIL-1 β :Caf1 stability and decrease in hIL-1 β :Caf1-induced toxicity. Clearly, the presence of Caf1M reduced the formation of toxic aggregates and promoted the folding of the chimera. Specific interaction of Caf1M with hIL-1 β :Caf1 was demonstrated by the identification of hIL-1 β :Caf1-Caf1M complexes following IEF. C-terminal peptides (14 amino acids) of this family of subunits are known to bind to the respective chaperone in a β -zipper interaction (9). Caf1M, however, was unable to promote the folding and solubilization of cytokine constructs possessing only the C-terminal 14 amino acids of the Caf1 subunit (data not shown). Resolution of the PapD chaperone-PapK subunit and FimC chaperone-FimH adhesin crystals (3, 28) has revealed that upon interacting with the C-terminal β -strand of the subunit, the chaperone completes an immunoglobulin fold of the subunit by temporarily donating its own G1 β -strand. Mutagenesis studies have provided evidence that the Caf1M chaperone interacts with the Caf1 subunit by a similar mechanism and hence most likely stabilizes the subunit by complementing an incomplete β -structure of the subunit (2, 22). Like the pilin subunits, a single Caf1 subunit would then be unable to form a compact globule and would become trapped on the surface of the plasma membrane. Only following completion of the subunit structure by donation of the Caf1M β -strand during Caf1-Caf1M complex formation would the Caf1 subunit fold correctly and be released from the membrane. In analogy to this, the energy released during the binding of Caf1M chaperone to the Caf1 domain of the chimeric proteins together with simultaneous folding of Caf1 seems to

FIG. 6. Hypothetical view of secretion of hIL-1 β :Caf1. The signal sequence directs hIL-1 β :Caf1 to the Sec general secretory pathway. Despite successful processing, hIL-1 β :Caf1 remains associated with the inner membrane [step 1, hIL-1 β , and spacer are in black, and Caf1 and the cleaved 35 S-Caf1-N(-2)D signal sequence are in gray]. Failure to form a soluble periplasmic protein, common to secreted hIL-1 β , hIL-1 α , and GM-CSF, is most likely due to an inability of the recombinant protein to fold at the surface of the inner membrane. Caf1M (M) specifically binds to the Caf1 domain of the chimera. Chaperone binding induces the folding of Caf1 (C) and causes dissociation of the chimera from the inner membrane (step 2) to the periplasm, where the hIL-1 β domain of the chimera (IL) is free to fold correctly (step 3). The resulting complex approaches the outer membrane channel formed by the Caf1A usher (A). It is likely that the chimera is secreted to the cell surface simultaneously with its assembly into linear polymers (step 4). The chaperone capping the Caf1 subunit is replaced by the Caf1 domain of a newly incorporating chimera. Most probably, this process occurs by the donor strand exchange mechanism (3, 28). According to this mechanism, the GI β -strand of the chaperone, complementing the Caf1 domain of the chimera, is replaced by the N-terminal sequence (gray) of the Caf1 part of the newly incorporating chimera (see text for details).

be sufficient for dissociation of the chimera from the inner membrane (Fig. 6, step 2). Apparently, the heterologous domain can then undergo spontaneous folding in the membrane-free environment (Fig. 6, step 3).

Fimbriae and capsules coat the bacterial surface with a very high copy number of a single protein. For this reason, they have frequently been investigated as choice carriers for the expression of heterologous epitopes (15, 25, 30, 33). In these previous studies, DNA-containing epitopes have been inserted in frame within the structural gene for the subunit. Due to strict limitations on permissible sites for insertion without disturbing fimbriae assembly, success has been limited to the insertion of very short epitopes (15). In contrast, this study shows promise that the Caf system can be adapted to the surface localization of entire proteins. When the hIL-1 β :Caf1-Caf1M complex accumulated in the periplasm, it polymerized with a regular banding pattern similar to that of the wild-type Caf1. This indicates that the presence of the N-terminal hIL-1 β did not block polymer formation of the Caf1 subunit. In the complete Caf system, polymerization of Caf1 most likely

occurs at the cell surface and provides energy for the outer membrane translocation step. In the presence of both the Caf1 A outer membrane protein and Caf1M chaperone, the hIL-1 β :Caf1 chimera could be detected at the cell surface of *E. coli*, indicating that Caf1M-mediated folding and polymerization of the chimera was following the native pathway. Each chaperone-usher system encodes its own specific outer membrane usher (29). Specificity is conferred by interaction with the subunit-chaperone complex, which hIL-1 β :Caf1 apparently still fulfills. The efficiency of the surface localization of hIL-1 β :Caf1, however, was rather low. This could be due to decreased efficiency at the level of targeting to the usher, to polymerization, or to size restrictions in the channel. Increase in production of surface chimera should be possible by optimizing key events at this stage. In support of this is the fact that the related composite pilin assembly systems are flexible with respect to the size of subunit assembled; hence, both the small pilin subunit and large adhesin are translocated via the usher (29).

Perhaps one of the most surprising aspects of this study is the fact that in the chimera, Caf1 polymerization apparently still occurred in the normal way. It has been proposed that during assembly of Pap and type I pili, the disordered N-terminal extension of the pilin subunit forms a β -strand and replaces the chaperone G1 β -strand of the neighboring subunit, thus maintaining the complete immunoglobulin fold of each subunit (3, 28). We have preliminary evidence from deletion mutagenesis that the N terminus of Caf1 mediates Caf1 polymerization (A. V. Zav'yalov, M. MacIntyre, V. P. Zav'yalov, and S. Knight, unpublished data). With the hIL-1 β :Caf1 chimera, Caf1 polymerizes and appears to assemble on the cell surface despite fusion of the N terminus to hIL-1 β . The spacer linking peptide, however, is very flexible and would appear to be sufficiently so to permit interaction of the Caf1 N terminus with a neighboring Caf1 subunit, as indicated in Fig. 6. This example of hIL-1 β :Caf1 assembly on the cell surface or as periplasmic polymers shows a potential approach for the construction of novel polymeric protein structures. Options would then be available for the isolation of recombinant protein from the periplasm, for exposure at the cell surface or for subsequent proteolytic cleavage to release the heterologous protein.

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