

Subtilase cytotoxin cleaves newly synthesized BiP and blocks antibody secretion in B lymphocytes

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Shiga-toxigenic *Escherichia coli* (STEC) use subtilase cytotoxin (SubAB) to interfere with adaptive immunity. Its inhibition of immunoglobulin secretion is both rapid and profound. SubAB favors cleavage of the newly synthesized immunoglobulin heavy chain-binding protein (BiP) to yield a C-terminal fragment that contains BiP's substrate-binding domain. In the absence of its regulatory nucleotide-binding domain, the SubAB-cleaved C-terminal BiP fragment remains tightly bound to newly synthesized immunoglobulin light chains, resulting in retention of light chains in the endoplasmic reticulum (ER). Immunoglobulins are thus detained in the ER, making impossible the secretion of antibodies by SubAB-treated B cells. The inhibitory effect of SubAB is highly specific for antibody secretion, because other secretory proteins such as IL-6 are released normally from SubAB-treated B cells. Although SubAB also causes BiP cleavage in HepG2 hepatoma cells, (glyco)protein secretion continues unabated in SubAB-exposed HepG2 cells. This specific block in antibody secretion is a novel means of immune evasion for STEC. The differential cleavage of newly synthesized versus "aged" BiP by SubAB in the ER provides insight into the architecture of the ER compartments involved.

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Abbreviations used: BiP, immunoglobulin heavy chain-binding protein; HUS, hemolytic uremic syndrome; NBD, nucleotide-binding domain; PDI, protein disulfide isomerase; SBD, substrate-binding domain; STEC, Shiga-toxigenic *Escherichia coli*; SubAB, subtilase cytotoxin; UPR, unfolded protein response.

Shiga-toxigenic *Escherichia coli* (STEC) are responsible for food poisoning outbreaks and can cause serious human gastrointestinal disease, sometimes leading to life-threatening complications such as hemolytic uremic syndrome (HUS; Nataro and Kaper, 1998; Paton and Paton, 1998; Bettelheim, 2007). Other than Shiga toxin, some STEC strains also produce subtilase cytotoxin (SubAB). SubAB is an AB₅ toxin consisting of a catalytic A subunit and five B subunits that form a pentameric ring responsible for binding to the receptor on the host cell surface. In mice, SubAB causes systemic organ failure that may ultimately result in death (Paton et al., 2004; Wang et al., 2007).

The A subunit of SubAB is a serine protease that specifically cleaves and inactivates immunoglobulin heavy chain-binding protein (BiP)/glucose-regulated protein 78, a heat shock protein 70 family member (Paton et al., 2006). Mutation of the Ser₂₇₂ residue to alanine in the Asp-His-Ser catalytic triad of the A subunit inactivates SubAB (Paton et al., 2004). This mutant version

of SubAB has been used for vaccination and elicits antibodies that protect against a challenge with native SubAB or SubAB-producing bacteria (Talbot et al., 2005). Cytotoxicity of SubAB has been convincingly demonstrated to result from the cleavage of BiP at a dileucine motif (Leu⁴¹⁷, Leu⁴¹⁸ in mouse BiP) because overexpression of BiP in which the SubAB cleavage site has been eliminated protects cells from SubAB-induced cytotoxicity (Paton et al., 2006).

BiP fulfills several essential functions in the ER. As a chaperone, BiP assists in the folding and assembly of nascent secretory proteins by binding to them transiently, and BiP remains associated with mutant misfolded proteins (Bole et al., 1986; Gething et al., 1986; Pelham, 1986). In addition, BiP may play a role in gating the Sec61 complex or translocon (Hamman et al.,

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1998), in translocation of nascent proteins across the ER membrane (Madack et al., 1999), in dislocation of misfolded proteins from the ER for degradation (Chillarón and Haas, 2000), and in activation of the unfolded protein response (UPR; Bertolotti et al., 2000; Shen et al., 2002). BiP contains a nucleotide-binding domain (NBD) at its N terminus and a substrate-binding domain (SBD) at its C terminus. A KDEL sequence at its C-terminal end ensures BiP's retention in the ER (Haas and Meo, 1988). SubAB inactivates BiP through proteolytic cleavage, which separates the N-terminal NBD from the C-terminal SBD (Paton et al., 2006). SubAB-mediated BiP inactivation has been linked to decreased virion assembly of human cytomegalovirus (Buchkovich et al., 2008), reduced ER-associated degradation of proteins (Lass et al., 2008), and induction of the UPR in various cell types (Takano et al., 2007; Hayakawa et al., 2008; Morinaga et al., 2008; Wolfson et al., 2008).

A primary target for SubAB may be the spleen. Mice injected with SubAB exhibit splenic atrophy and lose ~60% of spleen weight 3 d after injection (Paton et al., 2004; Wang et al., 2007). B cells represent the major lymphocyte population in the spleen responsible for secretion of antibodies, both so-called natural antibodies and those elicited by immunization. The B cell responds to encounter of its cognate antigen by ramping up the synthesis and secretion of immunoglobulins. BiP is a key player in assisting the folding and assembly of immunoglobulin heavy and light chains (Bole et al., 1986; Knittler and Haas, 1992). Thus, intoxication with SubAB and subsequent BiP cleavage could have a profound impact on the function of B cells, specifically with regard to immunoglobulin assembly and secretion. In addition, no BiP knock-out model is available that demonstrates whether BiP's function is indeed indispensable in B cells or whether BiP can be replaced, entirely or in part, by other chaperones. BiP is believed to also assist in the folding and assembly of other membrane or secreted proteins (Fourie et al., 1994; Gething and Sambrook, 1992; Gething, 1999; Ma and Hendershot, 2001), but there is no easy way to distinguish the client proteins that require BiP from those that do not.

Here, we describe the effect of SubAB on B cell function and show that it blocks secretion of immunoglobulins, but not of other proteins we examined. We propose a model that describes how SubAB targets newly synthesized BiP and generates a cleaved BiP fragment that preferentially sequesters the newly synthesized light chains. This causes a blockade in antibody secretion by activated B cells. Our results illustrate how pathogenic STEC subvert the host's immune system and do so rapidly.

RESULTS

BiP is the ER substrate for SubAB in B cells

To confirm that BiP is the substrate for SubAB (Paton et al., 2006) in mouse B cells, we treated B cells with SubAB and examined the toxin's effect on a select group of ER proteins, in addition to BiP itself. In all experiments, we used the catalytically inactive, mutant version of SubAB (Paton et al., 2006)

as a control. We found that BiP was cleaved into two fragments, representing the N-terminal NBD (~44 kD) and the C-terminal SBD (~28 kD) in SubAB-exposed B cells (Fig. 1 A). In contrast, protein disulfide isomerase (PDI), AAA ATPase (p97), calnexin, calreticulin, and ERdj3 were affected neither quantitatively nor qualitatively by exposure of B cells to SubAB. SubAB-mediated depletion of BiP induces an UPR in many cell types (Takano et al., 2007; Hayakawa et al., 2008;

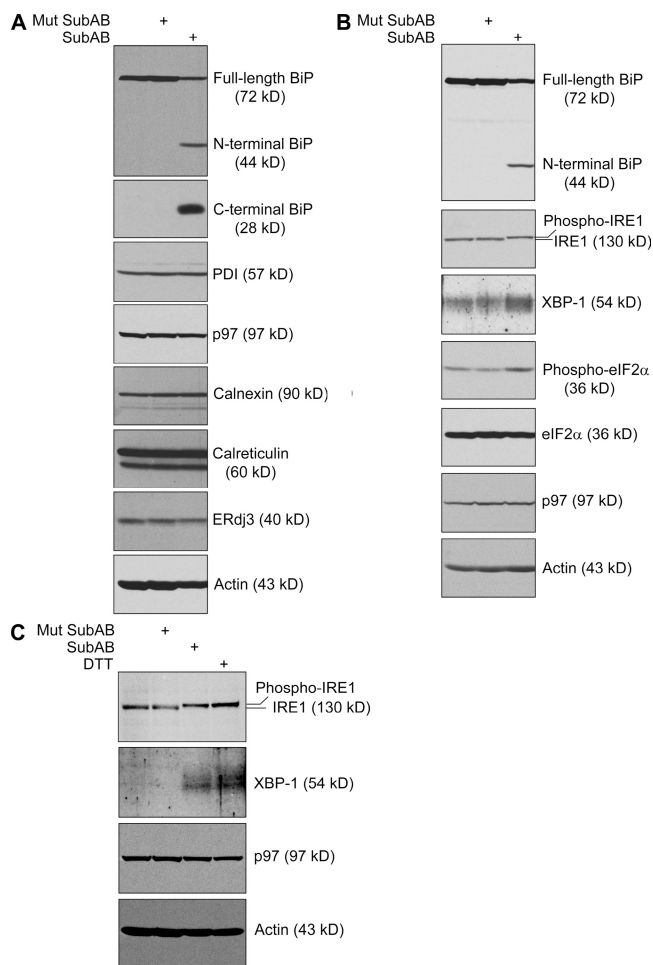


Figure 1. SubAB cleaves BiP and induces the UPR in B cells. (A) BiP is a specific target for SubAB in B cells. B cells purified from spleens of MD4 mice were cultured in LPS (20 μ g/ml) to induce differentiation. 3-day LPS-stimulated B cells were treated with 0.1 μ g/ml mutant or native SubAB for 3 h before lysis. Lysates were immunoblotted using antibodies against the BiP N terminus or C terminus; PDI; AAA-ATPase (p97); calnexin; calreticulin; ERdj3 (an HSP40 family protein); and actin. (B) IRE-1/XBP-1 and PERK pathways are activated in B cells in response to SubAB treatment. Similar lysates were immunoblotted using antibodies against BiP at its N terminus, IRE-1, XBP-1, phospho-eIF2 α , eIF2 α , p97, and actin. (C) 1-d LPS-stimulated B cells were treated with 0.1 μ g/ml mutant or native SubAB or dithiothreitol (DTT) for 3 h before lysis. Lysates were immunoblotted using antibodies against IRE-1, XBP-1, p97, and actin. Results shown in each panel are representative of three independent experiments. For each experiment, B cells were pooled from at least two mouse spleens.

Morinaga et al., 2008; Wolfson et al., 2008). In SubAB-exposed B cells, we observed a subtle yet clear increase in the apparent molecular weight of IRE-1, which is consistent with IRE-1 phosphorylation (Fig. 1, B and C). Upon SubAB treatment, the level of XBP-1-spliced protein is up-regulated in 1-d LPS-stimulated B cells, but only moderately so in 3-day LPS-stimulated B cells (Fig. 1, B vs. C), consistent with the fact that LPS itself is a potent UPR inducer for B cells (Reimold et al., 2001; Calfon et al., 2002). Activation of the PERK axis of the UPR was also examined. We found that phosphorylation of eIF2 α increases slightly in response to SubAB treatment in 3-day LPS-stimulated B cells (Fig. 1 B).

SubAB preferentially cleaves newly synthesized BiP

To address the kinetics of BiP cleavage by SubAB, we incubated 3-day LPS-stimulated B cells with SubAB for 0, 15, 30, 60, 120, and 180 min, and assessed BiP cleavage by immunoblotting of total cell extracts. We observed that cleavage of

BiP occurs within 15 min after addition of SubAB and reaches a plateau after a 30-min exposure (Fig. 2 A). The resultant C-terminal BiP fragment is stable throughout the course of the experiment, but the N-terminal BiP fragment is less so. The fate of the cleaved BiP fragments may include their removal from the ER, followed by degradation. We further explored the kinetics of BiP cleavage by pulse-chase analysis. We radiolabeled B cells for 4 h and chased them for 0, 15, 30, 60, 120, or 180 min in the presence of native or mutant SubAB. In B cells treated with mutant SubAB, BiP degrades slowly, with \sim 80% BiP remaining after the 180 min chase (Fig. 2 B), yielding a half-life in excess of 6 h, presumably reflecting the normal turnover rate of BiP. When treated with SubAB, \sim 60% BiP was already cleaved within 30 min. BiP cleavage beyond this time point is less dramatic (Fig. 2 B). We therefore wondered whether SubAB preferentially cleaves newly synthesized BiP, which is accessible to SubAB in the ER and presumably located at those sites where

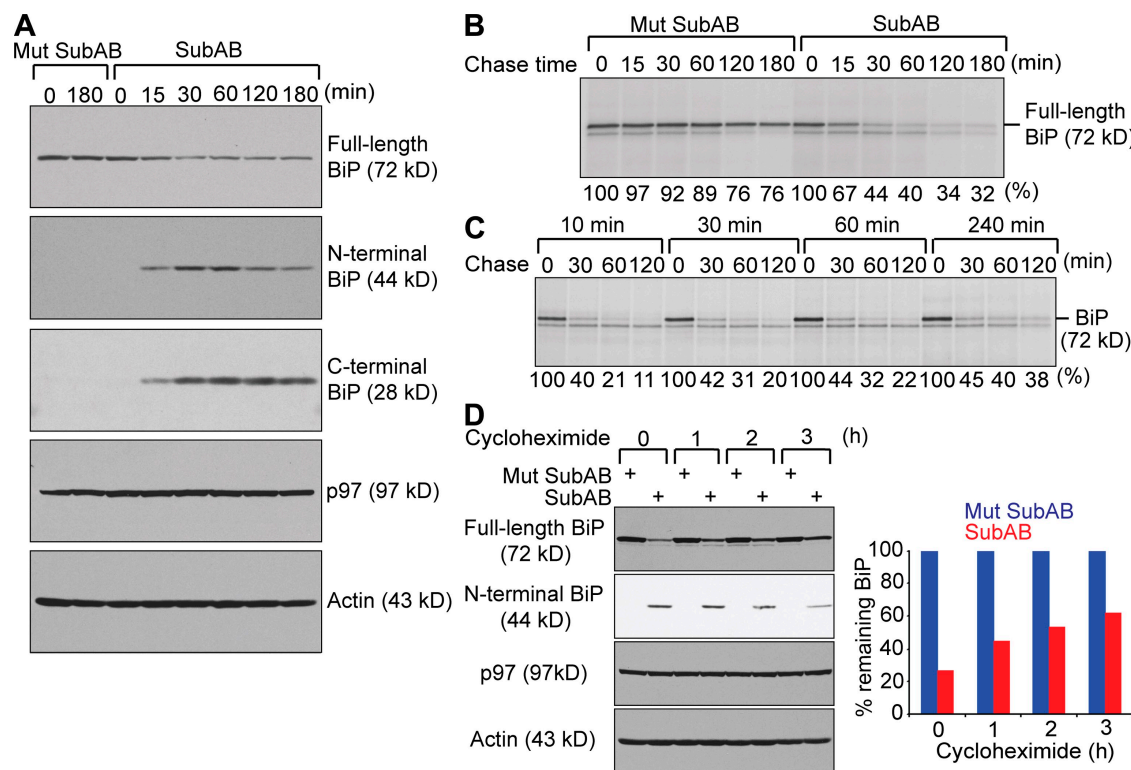


Figure 2. SubAB preferentially cleaves newly synthesized BiP in B cells. (A) BiP cleavage by SubAB reaches its maximum within 30 min. B cells purified from spleens of μ S $^{-/-}$ mice were cultured in LPS (20 μ g/ml) to induce differentiation. 3-day LPS-stimulated B cells were treated with 0.1 μ g/ml mutant or native SubAB for the times indicated before lysis. Lysates were immunoblotted for full-length BiP, N-terminal BiP, C-terminal BiP, p97, and actin. (B) 3-day LPS-stimulated μ S $^{-/-}$ B cells were labeled with [35 S]methionine and [35 S]cysteine for 4 h, chased for the indicated times in the presence of mutant or native SubAB, and lysed. Full-length BiP was immunoprecipitated from the lysates using an anti-KDEL antibody. Radioactive polypeptides were quantified using a phosphorimager. (C) Newly synthesized BiP is cleaved completely by SubAB. 3-day LPS-stimulated μ S $^{-/-}$ B cells were radiolabeled for 10, 30, 60, or 240 min, chased for the indicated times in the presence of SubAB, and lysed. Full-length BiP was immunoprecipitated from the lysates using the anti-KDEL antibody. Radioactive polypeptides were quantified using a phosphorimager. (D) Aged BiP is not cleaved efficiently by SubAB. 3-day LPS-stimulated B cells were pretreated with CHX (100 μ M) for 0, 1, 2, or 3 h and subsequently exposed to 0.1 μ g/ml mutant or native SubAB for 2 h before lysis. Lysates were immunoblotted for full-length BiP, N-terminal BiP, p97, and actin. Intensities of protein bands in the full-length BiP immunoblot were determined using ImageJ (National Institutes of Health) and data were plotted. Results shown in each panel are representative of three independent experiments. For each experiment, B cells were pooled from at least two mouse spleens.

proteins enter the ER. We therefore radiolabeled B cells for 10, 30, 60, and 240 min and chased these cells for 0, 30, 60, and 120 min in the presence of SubAB to examine whether newly synthesized BiP (10-min pulse) is indeed more sensitive to SubAB-mediated cleavage. All samples were normalized to equivalent amounts of radioactivity incorporated. Whereas newly synthesized BiP (10-min pulse) is cleaved by SubAB nearly completely within 2 h, BiP produced after 240 min of labeling is far more resistant to cleavage (Fig. 2 C). We pretreated B cells with cycloheximide to block protein synthesis, allowed the pool of BiP to age in the absence of ongoing protein synthesis, and then exposed the cells to SubAB. Cycloheximide-treated cells contain more BiP resistant to SubAB cleavage, consistent with the notion that newly synthesized BiP is the preferred target for SubAB (Fig. 2 D). All of these approaches yield a consistent result and lead us to the following conclusion: BiP found at sites in the ER where newly synthesized proteins are inserted is more readily cleaved than BiP that is given an opportunity to mature and move to more distal aspects of the ER. Although the entry pathway of SubAB remains to be explored in detail, our data suggest that delivery of SubAB targets those parts of the ER most directly involved in immunoglobulin folding/assembly/secretion.

SubAB blocks secretion of IgM and free κ light chains

To examine the effect of SubAB treatment on B cell function, we stimulated naive B cells with LPS for 3 d, and radiolabeled these cells in the presence of SubAB or the inactive mutant SubAB for 0, 20, 40, or 60 min. Total protein synthesis within the 20-min labeling period is not affected by SubAB. Compared with control cells treated with mutant SubAB, exposure to SubAB for 40 and 60 min reduces protein synthesis by no more than ~20 and ~40%, respectively (Fig. S1; Morinaga et al., 2008).

To examine the effect of SubAB on secretion, we exposed 3-day LPS-stimulated B cells to SubAB or to the inactive mutant SubAB for 30 min, a time frame within which cleavage of BiP by SubAB should have reached its maximum and protein synthesis is not significantly affected (Fig. 2, A and B, and Fig. S1). We radiolabeled these SubAB-exposed cells for 10 min, chased them for 2 h (Fig. S2 A), and then examined immunoglobulin secretion by immunoprecipitation. Secretion of IgM ceases after as little as 30 min of SubAB treatment (Fig. 3 A), coincident with intracellular retention of IgM (Fig. 3 B). Immunoprecipitates from the culture media using the anti- κ antibody contain not only assembled (to μ chain) but also free κ chains, explaining why a stronger κ signal was observed compared with immunoprecipitations performed with the anti- μ antibody (Fig. 3 A). The data suggest that free κ chains were likewise retained in SubAB-treated B cells. In μ S^{-/-} B cells, which make only membrane IgM and cannot secrete IgM, the secretion of free κ chains is indeed blocked by SubAB treatment (Fig. 3 C). HepG2 cells, a human hepatocellular carcinoma cell line that secretes a variety of serum proteins, including α 1-antitrypsin and albumin, were examined to see whether blocking of secretion is

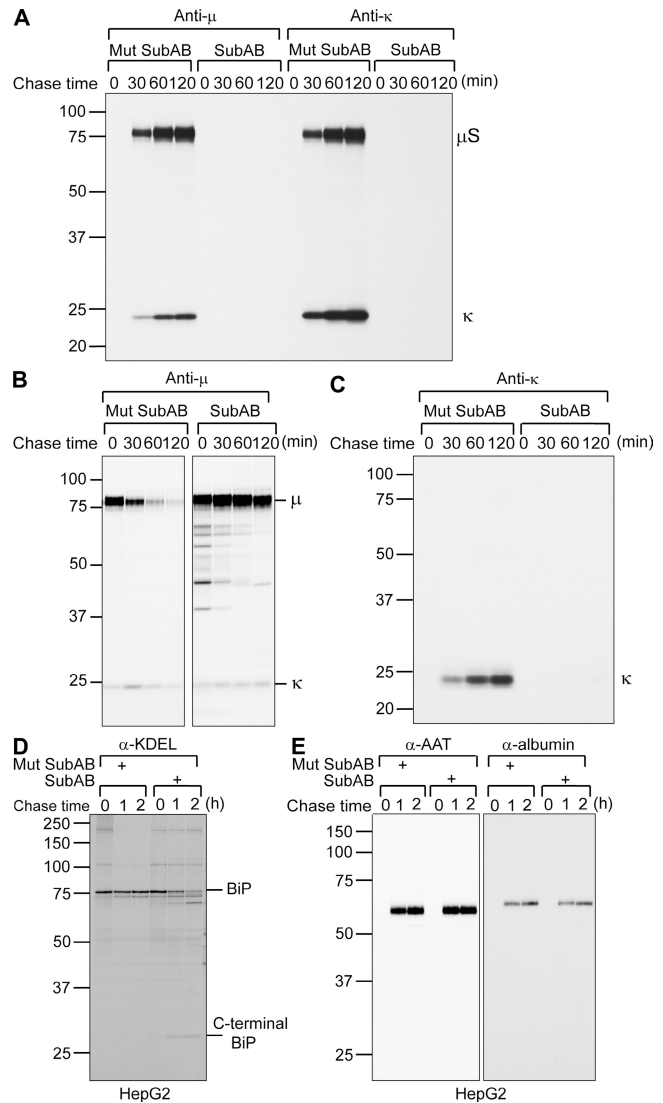


Figure 3. SubAB blocks secretion of IgM and free κ light chains.

(A) 3-day LPS-stimulated MD4 B cells were pretreated with mutant or native SubAB for 30 min, labeled with [³⁵S]methionine and [³⁵S]cysteine for 10 min, and chased for the indicated times. Secreted IgM was immunoprecipitated from the culture media using an anti- μ or - κ antibody. (B) 3-day LPS-stimulated MD4 B cells, treated and pulse-chased as described in A, were lysed. IgM was immunoprecipitated from the lysates using anti- μ antibody. A longer exposed autoradiogram is shown for SubAB-treated samples. (C) 3-day LPS-stimulated μ S^{-/-} B cells were pretreated with mutant or native SubAB for 30 min, radiolabeled for 10 min, and chased. The secreted free κ chains were immunoprecipitated from the culture media using anti- κ antibody. Approximately seven times as much starting material was used for immunoprecipitations for the SubAB-treated samples to emphasize the differences in levels of secretion. (D) HepG2 cells were treated with mutant or native SubAB during the 30-min radiolabeling period, and chased. Lysates were used for immunoprecipitation with an anti-KDEL antibody. (E) Culture media from toxin-treated, radiolabeled HepG2 cells were immunoprecipitated for α 1-antitrypsin (AAT) and albumin. Results shown in each panel are representative of three independent experiments. For each experiment shown in panels A–C, B cells were pooled from at least two mouse spleens.

a more general consequence of SubAB treatment. BiP is cleaved in SubAB-treated HepG2 cells to an extent similar to that seen in B cells (Fig. 3 D), but these cells still secrete

α 1-antitrypsin and albumin into the media at their usual rates and quantities (Fig. 3 E). BiP cleavage by SubAB thus does not lead to a general block of the secretory pathway.

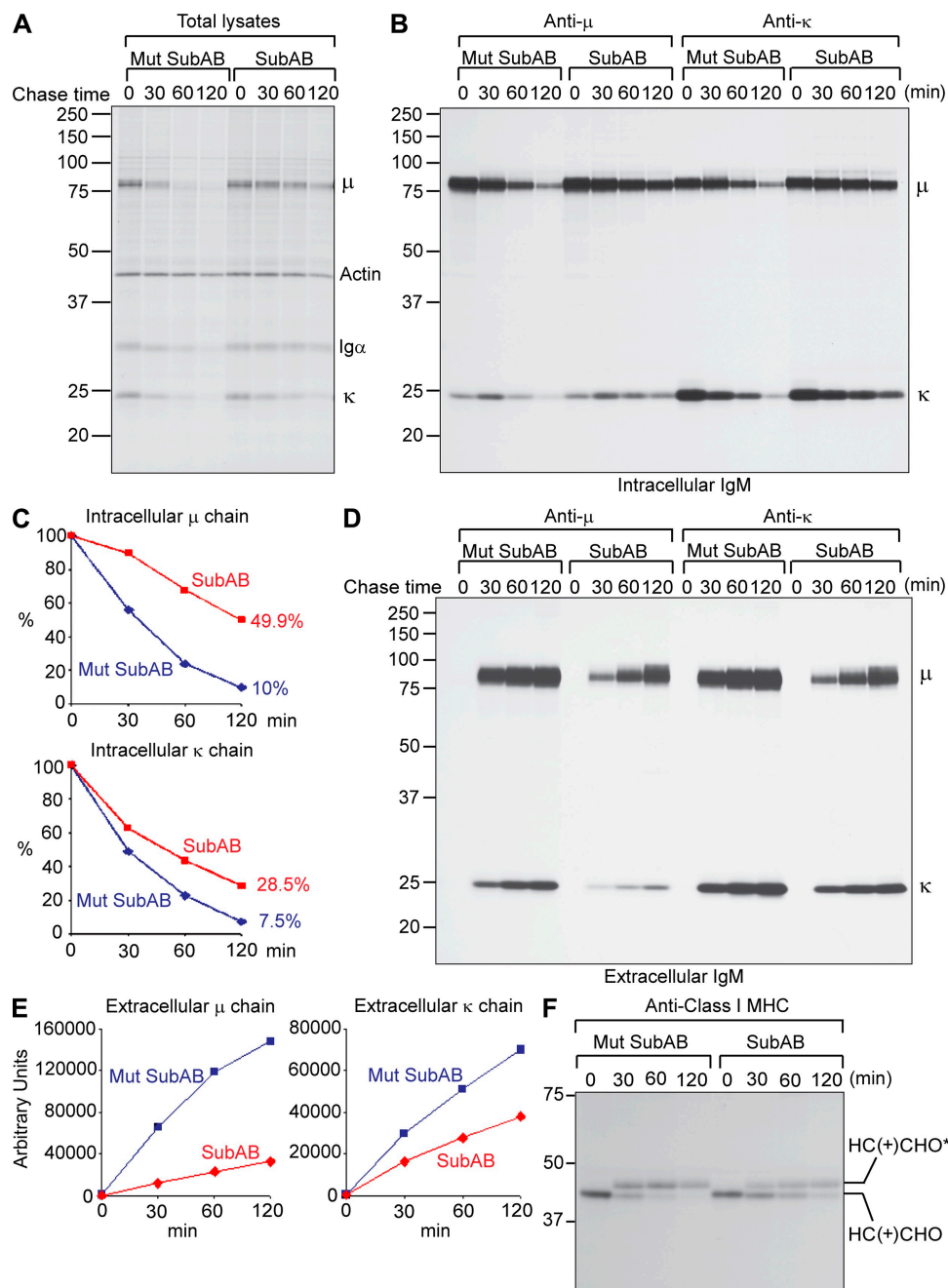


Figure 4. SubAB blocks secretion of IgM, but not intracellular transport of class I MHC molecules. (A) 3-day LPS-stimulated MD4 B cells were labeled with [35 S]methionine and [35 S]cysteine for 10 min in the presence of mutant or native SubAB, chased for the indicated times, and lysed. Total lysates were analyzed by SDS-PAGE. (B) Lysates (A) were used for immunoprecipitations with antibodies against μ or κ . (C) Band intensities of μ (immunoprecipitated with α - μ) and κ (immunoprecipitated with α - κ) were quantified using a phosphorimager, and the ratio was determined by comparing to the total μ or κ signal at time point zero. (D) 3-day LPS-stimulated B cells were radiolabeled for 10 min in the presence of mutant or native SubAB, and chased for the indicated times. Culture media from each chase point were used for immunoprecipitations with antibodies against μ or κ . (E) Band intensities of μ (immunoprecipitated with α - μ) and κ (immunoprecipitated with α - κ) were quantified using a phosphorimager, and the obtained raw numbers were plotted. (F) Lysates (A) were also used for immunoprecipitations of the class I MHC heavy chains. CHO, high-mannose glycans; CHO*, complex-type glycans. Results shown in A, B, D, and F are representative of three independent experiments. For each experiment shown in A, B, D, and F, B cells were pooled from at least two mouse spleens.

To examine in greater detail the kinetics of the blockade in IgM secretion, we used a different pulse-chase protocol, because the 30-min treatment with SubAB already completely blocks IgM secretion. We therefore radiolabeled LPS-stimulated B cells in the presence of SubAB for only 10 min, and then chased these cells for 2 h in the continued presence of SubAB (Fig. S2 B). In total cell lysates, we identified four prominent bands that correspond to μ heavy chain, actin, Ig α , and κ light chain (Fig. 4 A). When their intensities were compared at the zero time point, none of them were affected by SubAB. Both μ and κ persisted in the lysates from SubAB-treated B cells at all chase times (Fig. 4 A), consistent with a blockade of IgM secretion, as confirmed by immunoprecipitation of μ and κ from cell lysates and culture supernatants (Fig. 4, B–E). Approximately 90% IgM was found to be secreted by mutant SubAB-treated B cells after a 2-h chase, but only ~50% IgM was secreted from SubAB-treated B cells (Fig. 4, B and C), although exposure to SubAB was for 10 min only. Although the anti- μ antiserum immunoprecipitates only κ chains that have been assembled with μ chains, the anti- κ antiserum immunoprecipitates the assembled as well as the free κ chains (Fig. 4, B and D). Secretion of κ chains as examined by immunoprecipitations using the anti- κ antiserum is blocked even when B cells were exposed to

SubAB for only 10 min in the course of radiolabeling (Fig. 4, B–E). Similar results were obtained for IgM with λ light chains, obtained from κ chain knockout ($\kappa^{-/-}$) B cells (Fig. S3). We then examined whether transport of class I MHC molecules to the cell surface was affected by exposure to SubAB. Similar amounts of class I MHC molecules in control versus SubAB-treated B cells acquire complex-type N-linked glycans (Fig. 4 F). SubAB-treated B cells contain higher levels of high-mannose-carrying class I MHC (the ER form) when compared at the 30- and 60-min chase points, consistent with at least some perturbation of ER functions (Lass et al., 2008).

SubAB blocks the ER-exit of membrane IgM, but not of class I MHC molecules

We next investigated the trafficking of membrane IgM and class I MHC products using $\mu S^{-/-}$ B cells. These cells do not produce secreted IgM, and therefore allow an easy electrophoretic distinction between high-mannose- and complex-type glycan-carrying membrane IgM (Boes et al., 1998; Hu et al., 2009). In cells exposed to SubAB treatment for 30 min, class I MHC molecules still acquire complex-type glycans (Fig. 5 B), but acquisition of complex-type glycans by membrane IgM is blocked completely (Fig. 5 A), indicating that the latter is trapped in the ER.

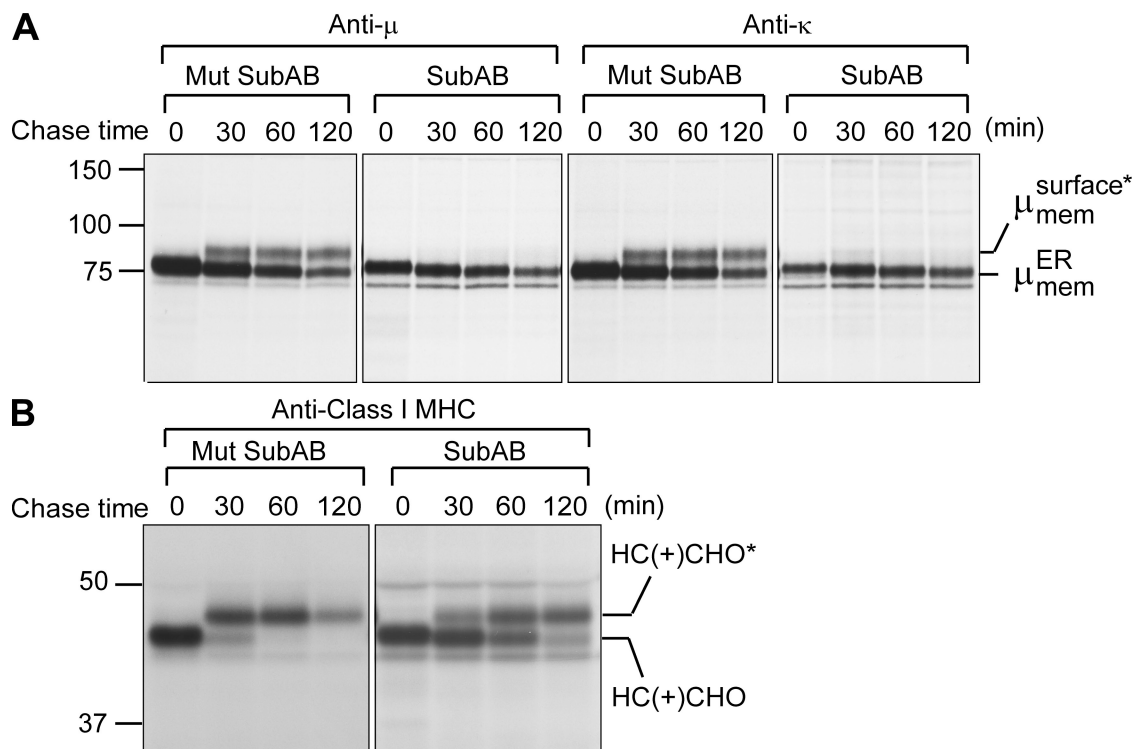


Figure 5. SubAB blocks the ER-exit of membrane IgM, but not class I MHC molecules. 3-day LPS-stimulated $\mu S^{-/-}$ B cells were pretreated with mutant or native SubAB for 30 min, labeled with [35 S]methionine and [35 S]cysteine for 10 min, chased, and lysed. Membrane IgM was immunoprecipitated from lysates using anti- μ and - κ antibodies (A). Class I MHC molecules were immunoprecipitated using an anti-class I heavy chain antibody (B). Asterisk represents molecules bearing complex-type glycans. Longer exposed gels are shown for SubAB-treated samples to emphasize the absence of complex-type glycan modification on IgM. Results shown in each panel are representative of three independent experiments. For each experiment, B cells were pooled from at least two mouse spleens.

SubAB blocks IgM secretion by sequestration of newly synthesized light chains in the ER

In mice, 90–95% of B cells synthesize κ light chains, and the remainder express λ light chains. Although free κ and λ light chains can exit the ER and complete the secretory pathway, exit of μ heavy chains from the ER requires their correct assembly with light chains (Vanhove et al., 2001). Because secretion of free κ chains in MD4 and $\mu S^{-/-}$ B cells is blocked by SubAB (Figs. 3 A and 3C), the failure of μ heavy chains to leave the ER might be caused by retention of κ chains, and therefore also of the μ chains bound to them. The behavior of the κ chain may thus hold the key to understanding how SubAB affects IgM secretion. Because BiP is the only known substrate for SubAB in the ER (Paton et al., 2006; Fig. 1), we hypothesized that SubAB-mediated BiP cleavage products might trap κ chains in the ER, although a direct effect of BiP cleavage on immunoglobulin heavy chains remains a possibility as well.

We first labeled LPS-activated B cells for 4 h with [35 S]methionine and [35 S]cysteine to allow robust labeling of BiP. We then treated cells with SubAB for 2 h during the chase (Fig. S2 C). We used an anti-KDEL antibody to immunoprecipitate BiP and observed that the anti-KDEL antibody immunoprecipitated both intact BiP and the C-terminal BiP fragment (Fig. 6 A). Indeed, when BiP is cleaved, its C-terminal fragment is found in a complex with κ chains (Fig. 6, A and B). Similar association occurs between the C-terminal BiP fragment and λ chains when $\kappa^{-/-}$ B cells are exposed to SubAB (Fig. S4). BiP favors binding to newly synthesized κ chains (Knittler and Haas, 1992). To investigate how SubAB-cleaved C-terminal BiP fragment acts on newly synthesized κ chains, we examined κ chains synthesized during a 10-min pulse. Intact BiP binds only weakly to newly synthesized κ chains (Fig. 6 C), consistent with a transient interaction between BiP and κ chains (Knittler and Haas, 1992;

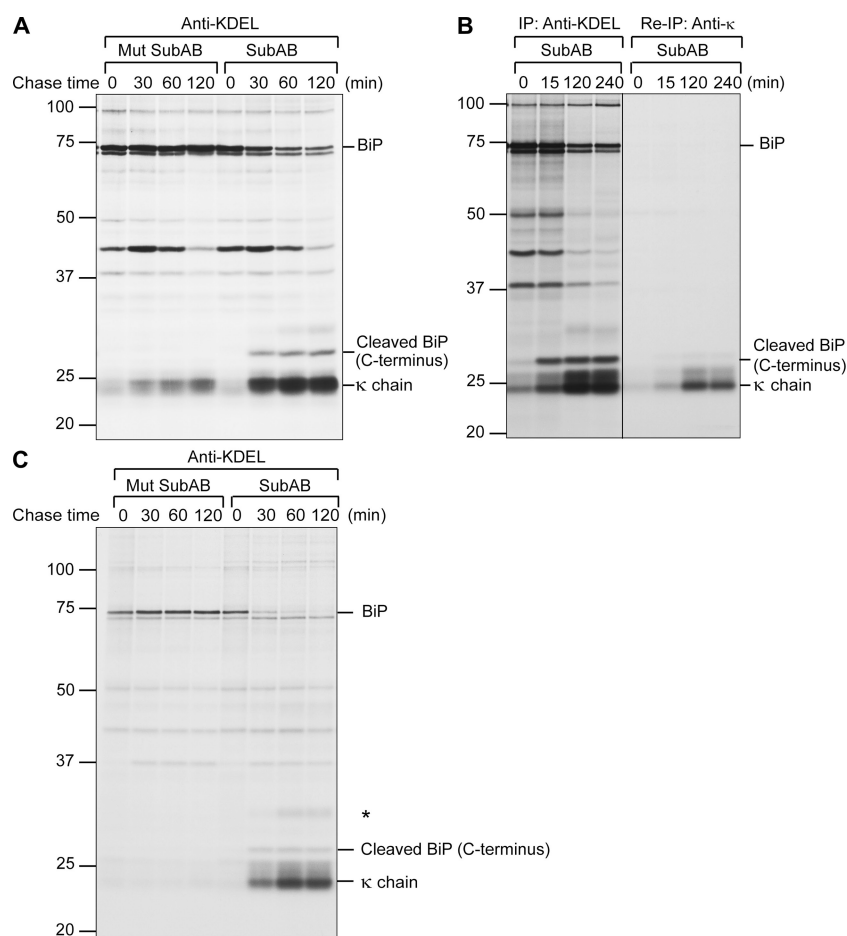


Figure 6. The C-terminal BiP cleavage fragment retains κ light chains in the ER. (A) 3-day LPS-stimulated $\mu S^{-/-}$ B cells were labeled with [35 S]methionine and [35 S]cysteine for 4 h, washed with PBS, and treated with mutant or native SubAB for the indicated times. Immunoprecipitations were performed using an anti-KDEL antibody. In addition to BiP, the anti-KDEL antibody retrieves additional polypeptides, but none of these are affected by SubAB. (B) In a similar but independent experiment, proteins immunoprecipitated with the anti-KDEL antibody were eluted and reimmunoprecipitated with an anti- κ antibody. (C) 3-day LPS-stimulated $\mu S^{-/-}$ B cells were labeled with [35 S]methionine and [35 S]cysteine for 10 min and chased in the presence of mutant or native SubAB for the indicated times. Lysates were immunoprecipitated with the anti-KDEL antibody. Asterisk marks an unidentified polypeptide. Results shown in each panel are representative of three independent experiments. For each experiment, B cells were pooled from at least two mouse spleens.

Knittler et al., 1995; Skowronek et al., 1998). However, the SubAB-cleaved C-terminal BiP fragment binds strongly to these newly synthesized κ or λ chains (Fig. 6 C and Fig. S5). Binding of the C-terminal BiP fragment to κ chains must be

of high affinity, because the BiP- κ complex survives immunoprecipitation in a buffer containing 0.1% SDS and is stable in cells for at least 4 h (Fig. 6 B). This result explains why no secretion of κ chains was observed from SubAB-treated

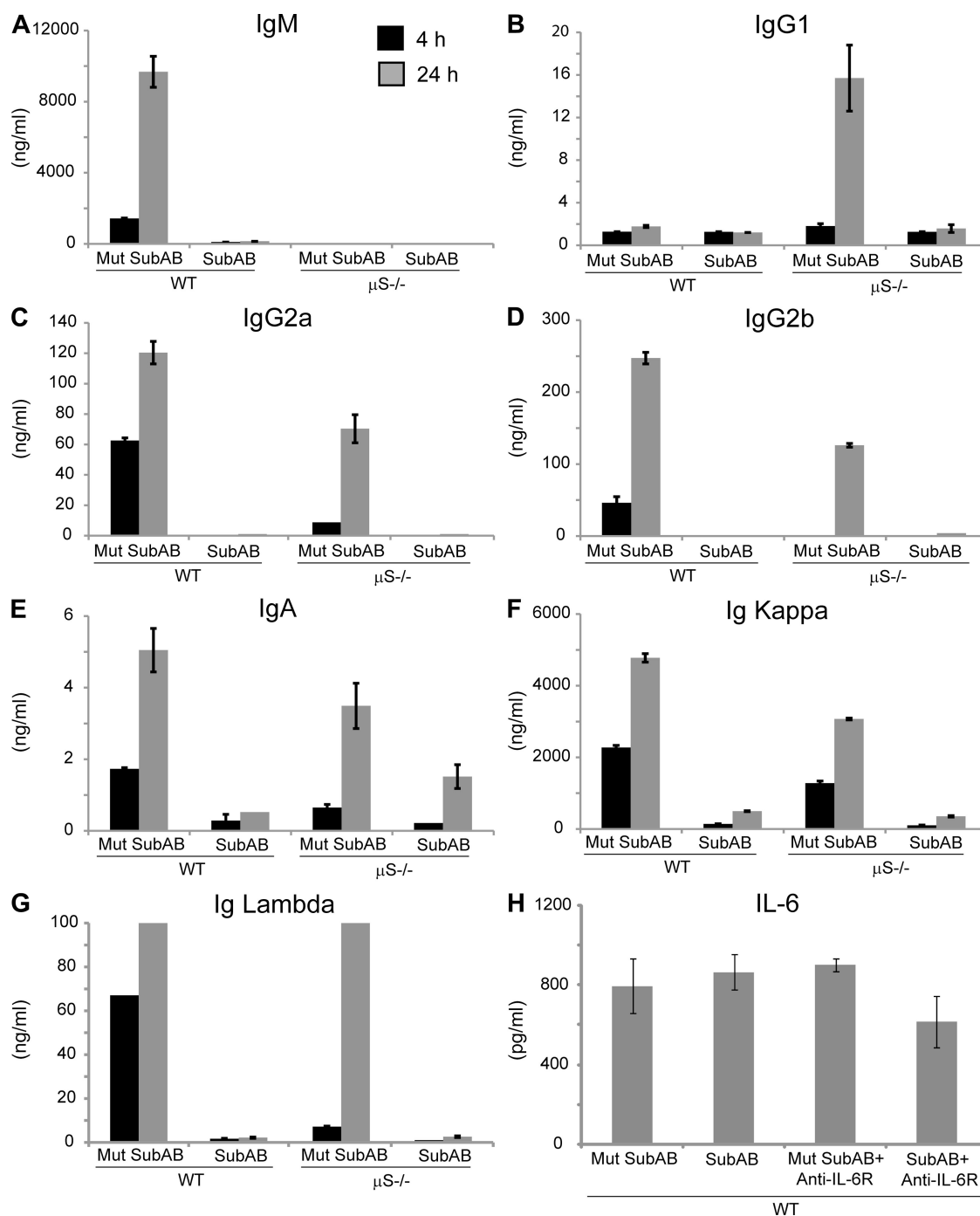


Figure 7. SubAB blocks the secretion of antibodies, but not of IL-6. 3-day LPS-stimulated wild-type and $\mu S^{-/-}$ B cells were washed, counted, aliquoted into 96-well plates with fresh media containing mutant or native SubAB, and incubated for 4 h (black bars) or 24 h (gray bars). The levels of secreted IgM (A), IgG1 (B), IgG2a (C), IgG2b (D), IgA (E), Ig κ (F), or Ig λ (G) in culture media at each time point were determined by ELISA. Separate aliquots of cells were treated for 24 h with SubAB plus an antibody that blocks IL-6 receptor (IL-6R) to prevent internalization of secreted IL-6 via the IL-6R. The levels of IL-6 in culture media were measured by ELISA (H). Results (means \pm SD) shown in each panel are representative of two independent experiments. For each experiment, B cells were pooled from two spleens of each genotype.

B cells (Fig. 3, A and C). None of the available antibodies against BiP that are suitable for immunoblotting immunoprecipitate the N-terminal BiP fragment, making the characterization of its associated molecules impossible at this time. However, we could show indirectly that κ and μ chains bind only to the full-length BiP and C-terminal BiP fragment, but not to the N-terminal BiP fragment (Fig. S6).

SubAB blocks secretion of antibodies of various isotypes, but not IL-6 secretion

We propose that SubAB blocks IgM secretion by sequestering κ and λ chains via the C-terminal BiP fragment. If correct, secretion of antibodies of isotypes other than IgM should likewise be affected. We examined B cells from wild-type and $\mu S^{-/-}$ mice for the evidence of altered antibody secretion by ELISA after SubAB treatment. Although $\mu S^{-/-}$ mice cannot secrete IgM, they can still class switch to other isotypes that yield secreted immunoglobulins. Consistent with the aforementioned results, secretion of IgM, IgG1, IgG2a, IgG2b, IgA, Ig κ , and Ig λ were blocked in SubAB-treated B cells (Fig. 7, A–G). As a control, we examined the secretion by B cells of an immunoglobulin-unrelated protein, IL-6. Given that B cells respond to IL-6 in autocrine fashion, we also treated B cells with a blocking antibody to the IL-6 receptor to more accurately assess the amount of IL-6 secreted without the confounding effect of reabsorption. We found that IL-6 secretion is normal in SubAB-treated B cells (Fig. 7 H).

DISCUSSION

SubAB specifically cleaves BiP and deprives BiP of its function rapidly (Paton et al., 2006). We assessed the extent of cleavage of BiP by exposing LPS-stimulated B cells to SubAB for various lengths of time. The extent of BiP cleavage by SubAB is never complete in intact cells (Fig. 1, A and B, and Fig. 2 A). However, newly synthesized BiP is nearly completely susceptible to cleavage, whereas the cleaved fraction of BiP measured at steady state never exceeded 75% (Fig. 2, B–D). This result suggests the existence of distinct pools of BiP as defined by their susceptibility to SubAB cleavage. Newly synthesized BiP that must have remained close to the site at which insertion into the ER had occurred is fully susceptible to cleavage by SubAB, whereas the cleavage-resistant BiP presumably has moved beyond the reach of SubAB. This would explain how secretion of newly synthesized immunoglobulins can be blocked completely by SubAB without the need for quantitative cleavage of BiP in the ER. If the remaining 25% of intact BiP were to remain available for immunoglobulin assembly, it is difficult to envision why secretion should not continue, albeit at a reduced rate. The notion of functional heterogeneity in the ER is implicit in, for example, the existence of smooth and rough versions of the ER, but few other aspects of cellular physiology have been attributed to distinct subregions of the ER. Biosynthetic aspects of ER function, such as membrane insertion, disulfide bond formation, and glycosylation might well be relegated to areas of the ER distinct from those involved in quality con-

trol, including dislocation of misfolded proteins from the ER. SubAB uses $\alpha 2\beta 1$ integrin as a receptor to enter cells (Yahiro et al., 2006) and is transported to the ER by a clathrin-dependent retrograde pathway (Chong et al., 2008). Because SubAB reaches the ER by retrograde transport, it might arrive preferentially in those ER subregions dedicated to biosynthetic activities and fail to reach all subdivisions of the ER equally efficiently, even though BiP might be present at those locations at steady-state. This would explain the discrepancy between the extent of BiP cleavage and the extent of inhibition of immunoglobulin secretion and emphasize the utility of SubAB to explore the ER physiology.

BiP is one of several chaperones that assist protein folding in the ER. SubAB presents a unique tool for functional elimination of BiP in the absence of a (conditional) knockout allele in mice, thus creating an opportunity to investigate BiP functions in different cell types (Paton et al., 2006; Buchkovich et al., 2008; Lass et al., 2008; Morinaga et al., 2008; Wolfson et al., 2008). BiP binds immunoglobulin heavy chains produced by B cells (Haas and Wabl, 1983), and its cleavage by SubAB profoundly affects B cell function. Surface display of membrane IgM is blocked by SubAB treatment, and SubAB does so by causing retention of IgM in the ER (Fig. 5 A). A different type I integral membrane protein, the class I MHC molecule, is displayed at the B cell surface with a slight delay, but no signs of complete inhibition of intracellular transport (Fig. 5 B). SubAB-treated B cells continue to secrete IL-6 (Fig. 7 H), and the assembly of Ig α with Ig β is not affected (Fig. S7). Folding or assembly of IL-6, class I MHC molecules, Ig α and Ig β must therefore be largely BiP-independent. Even though secretion of immunoglobulins is blocked, the function of the secretory pathway remains largely intact.

How does the C-terminal BiP fragment retain light chains in the ER? Substrate binding at the C-terminus of HSP70 is tightly regulated by its NBD at the N-terminus (Schmid et al., 1994; Greene et al., 1995; Zhu et al., 1996; Voisine et al., 1999). The structure of a functionally intact bovine Hsc70, containing both the NBD and the SBD, shows the interaction between the two domains (Jiang et al., 2005). Likewise, BiP contains an SBD and a regulatory NBD, and introduction of the mutation R197E in the NBD of BiP compromises NBD–SBD domain interactions and substrate release from the SBD (Awad et al., 2008). Intact BiP binds only transiently to light chains (Knittler and Haas, 1992; Knittler et al., 1995; Skowronek et al., 1998). However, the SubAB-cleaved C-terminal BiP fragment, which contains only the SBD, binds strongly and stably to newly synthesized light chains (Fig. 6 C and Fig. S5). We propose the following model for how sequestration of light chains by SubAB may occur (Fig. 8). (a) BiP binds to nascent light chains through its C-terminal SBD, and normal cycles of ATP binding/hydrolysis in the NBD mediate conformational changes in the SBD, allowing transient interaction between BiP and light chains. (b) SubAB cleaves BiP. With the loss of its NBD, the C-terminal SBD undergoes the usual conformational change, and its association with the light chain is now firmly locked in. Because

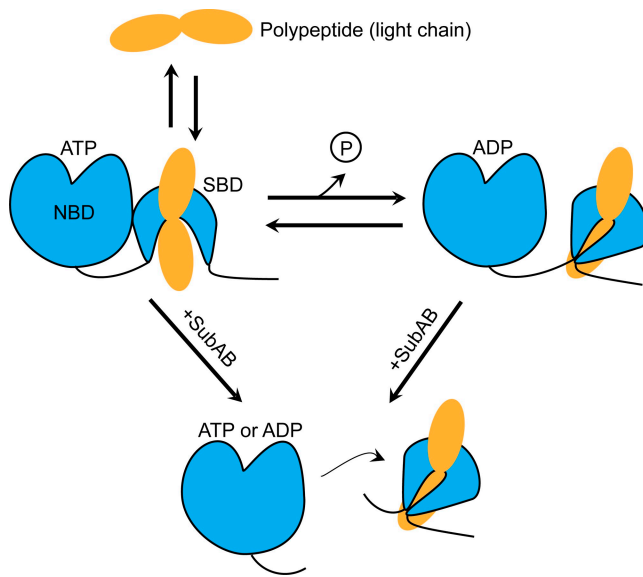


Figure 8. A model for the mode of action of SubAB. SubAB cleaves BiP into two segments, one containing the NBD, and the other containing the SBD. The SBD consists of short stretches of hydrophobic amino acids that allow its binding to unfolded/misfolded proteins. SubAB cleaves BiP, resulting in sequestration of immunoglobulin light chains through interactions with its cleaved product, the SBD of BiP.

substrate release from the SBD requires ATP binding to the NBD, which is absent from the cleaved form of BiP, the SubAB-cleaved C-terminal SBD remains tightly associated with light chains as well as heavy chains. (c) Immunoglobulin heavy and light chains are thus sequestered by the C-terminal BiP fragment. The C-terminal BiP-bound κ and λ light chains likely recycle between the ER and the Golgi apparatus via the KDEL receptor.

STEC cause human gastrointestinal diseases, which in serious cases can lead to systemic complications such as HUS (Nataro and Kaper, 1998; Paton and Paton, 1998) and splenic atrophy (Paton et al., 2004; Wang et al., 2007). SubAB produced by STEC is sufficient to cause such a syndrome (Paton et al., 2004; Wang et al., 2007). Here, we show that STEC use SubAB to cause retention of κ and λ light chains and their associated heavy chains in the ER (Figs. 6; 7, F and G; S4; and S5). By sequestering both κ and λ light chains, SubAB inhibits secretion of antibodies of all isotypes, including IgA (Fig. 7 E). The protective function of IgA-producing B cells, primarily found in gut-associated lymphoid tissues, will thus be compromised. Inactivation of IgA-producing B cells in the gut may be beneficial to STEC to allow colonization. HUS usually develops in the late stages of STEC-caused gastrointestinal diseases; nevertheless, once SubAB enters the blood and makes its way to the spleen or bone marrow, all B cells will cease secreting antibodies. This specific block in antibody secretion is an obvious means of immune evasion for STEC. Our finding provides a rational explanation for the observation that calves infected by STEC quickly lose their Shiga toxin-specific antibodies in the sera (Fröhlich et al., 2009).

SubAB produced by STEC is a versatile tool to study the details of how BiP interceded in immunoglobulin folding and secretion. A detailed analysis of the biochemical properties of the BiP cleavage fragments produced by SubAB may provide mechanistic insight in how exactly BiP carries out its functions.

MATERIALS AND METHODS

Mice. Wild-type C57BL/6, MD4 (Goodnow et al., 1988), and μ S^{-/-} (Boes et al., 1998) mice are maintained in our laboratory. All animal protocols were approved by the Massachusetts Institute of Technology Committee on Animal Care. We thank K. Rajewsky (Harvard Medical School, Boston, MA) for providing us with spleens from κ ^{-/-} mice (Zou et al., 1993).

Antibodies and reagents. Antibodies to N-terminal BiP (Cell Signaling Technology), C-terminal BiP (Stressgen), KDEL (Stressgen), p97 (Fitzgerald), calreticulin (Stressgen), ERdj3 (Santa Cruz Biotechnology, Inc.), human albumin (Sigma-Aldrich), α 1-antitrypsin (Novus), actin (Sigma-Aldrich), IRE-1 α (Cell Signaling Technology), XBP-1 (Santa Cruz Biotechnology, Inc.), phospho-eIF2 α (Cell Signaling Technology), and e-IF2 α (Cell Signaling Technology), μ (SouthernBiotech), and κ (SouthernBiotech) were obtained commercially. Antibodies against class I MHC heavy chain (p8) and PDI were produced in our laboratory. Anti-calnexin antibody was provided by D.B. Williams (University of Toronto, Toronto, Canada). The following antibodies for ELISA were obtained from BD: IgM, IgG1, IgG2a, IgG2b, IgA, Igk, and Ig λ . The ELISA kit for detection of IL-6 was purchased from BD. ELISA plates were read using SpectraMax M2 microplate reader (Molecular Devices). LPS and cycloheximide were procured from Sigma-Aldrich. SubAB and its nontoxic mutant SubA_{A272}B were purified as previously described (Paton et al., 2004; Talbot et al., 2005).

Cell culture. Naive B lymphocytes were purified from mouse spleen by magnetic depletion of CD43-positive cells (Miltenyi Biotec). Naive B cells were cultured in RPMI 1640 media containing 10% FBS with or without LPS (20 μ g/ml).

SDS-PAGE and immunoblot. B cells were treated with SubAB (0.1 μ g/ml) for the indicated times and lysed in conventional RIPA buffer supplemented with protease inhibitors (Calbiochem). Lysates were cleared at 16,000 g for 10 min at 4°C, resolved by SDS-PAGE (10% acrylamide), and electrophoretically transferred onto a nitrocellulose membrane, which was then blocked with 5% nonfat milk in PBS-T (PBS containing 0.05% Tween 20, pH 7.4) before incubation with a primary antibody. After incubating with horseradish peroxidase (HRP)-conjugated secondary antibody (SouthernBiotech), the PBS-T-washed membrane was developed using the Western Lightning Chemiluminescence Reagent PLUS system (PerkinElmer).

Pulse-chase labeling and immunoprecipitation. 3-day LPS-stimulated B cells were starved in methionine- and cysteine-free media containing dialyzed serum for 1 h, and then pulse labeled for 10 min with 250 μ Ci/ml of [³⁵S]methionine and [³⁵S]cysteine in the presence of SubAB or its nontoxic mutant counterpart. In some experiments, cells were radiolabeled for 4 h or treated with SubAB before pulse labeling or only during the chase period, as indicated in the figure legends. At the end of each chase point, cells were rinsed twice with PBS and lysed in conventional RIPA buffer containing protease inhibitors. Pre-cleared lysates were incubated with a primary antibody and protein G-agarose beads, washed, eluted from the beads using reducing Laemmli SDS-PAGE sample buffer, and analyzed by SDS-PAGE and fluorography. Quantitation was performed by phosphorimaging.

Online supplemental material. Fig. S1 shows that prolonged exposure to SubAB inhibits protein synthesis in B cells. Fig. S2 summarizes the radiolabeling strategies. Fig. S3 shows that SubAB blocks secretion of IgM containing λ light chains. Fig. S4 shows that the SubAB-cleaved C-terminal BiP

fragment sequesters not only κ but also λ light chains. Fig. S5 shows that the C-terminal BiP cleavage fragment retains newly synthesized κ and λ light chains in the ER. Fig. S6 shows that free κ chains interact with full-length and C-terminal, but not N-terminal BiP. Fig. S7 shows that SubAB does not affect the assembly of Ig α with Ig β . Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20090782/DC1>.

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REFERENCES

- Awad, W., I. Estrada, Y. Shen, and L.M. Hendershot. 2008. BiP mutants that are unable to interact with endoplasmic reticulum DnaJ proteins provide insights into interdomain interactions in BiP. *Proc. Natl. Acad. Sci. USA*. 105:1164–1169. doi:10.1073/pnas.0702132105
- Bertolotti, A., Y. Zhang, L.M. Hendershot, H.P. Harding, and D. Ron. 2000. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat. Cell Biol.* 2:326–332. doi:10.1038/35014014
- Bettelheim, K.A. 2007. The non-O157 shiga-toxicogenic (verocytotoxigenic) *Escherichia coli*; under-rated pathogens. *Crit. Rev. Microbiol.* 33:67–87. doi:10.1080/10408410601172172
- Boes, M., C. Esau, M.B. Fischer, T. Schmidt, M. Carroll, and J. Chen. 1998. Enhanced B-1 cell development, but impaired IgG antibody responses in mice deficient in secreted IgM. *J. Immunol.* 160:4776–4787.
- Bole, D.G., L.M. Hendershot, and J.F. Kearney. 1986. Posttranslational association of immunoglobulin heavy chain binding protein with nascent heavy chains in nonsecreting and secreting hybridomas. *J. Cell Biol.* 102:1558–1566. doi:10.1083/jcb.102.5.1558
- Buchkovich, N.J., T.G. Maguire, Y. Yu, A.W. Paton, J.C. Paton, and J.C. Alvine. 2008. Human cytomegalovirus specifically controls the levels of the endoplasmic reticulum chaperone BiP/GRP78, which is required for virion assembly. *J. Virol.* 82:31–39. doi:10.1128/JVI.01881-07
- Calfon, M., H. Zeng, F. Urano, J.H. Till, S.R. Hubbard, H.P. Harding, S.G. Clark, and D. Ron. 2002. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature*. 415:92–96. doi:10.1038/415092a
- Chillarón, J., and I.G. Haas. 2000. Dissociation from BiP and retrotranslocation of unassembled immunoglobulin light chains are tightly coupled to proteasome activity. *Mol. Biol. Cell.* 11:217–226.
- Chong, D.C., J.C. Paton, C.M. Thorpe, and A.W. Paton. 2008. Clathrin-dependent trafficking of subtilase cytotoxin, a novel AB5 toxin that targets the endoplasmic reticulum chaperone BiP. *Cell. Microbiol.* 10:795–806. doi:10.1111/j.1462-5822.2007.01085.x
- Fourie, A.M., J.F. Sambrook, and M.J. Gething. 1994. Common and divergent peptide binding specificities of hsp70 molecular chaperones. *J. Biol. Chem.* 269:30470–30478.
- Fröhlich, J., G. Baljer, and C. Menge. 2009. Maternally and naturally acquired antibodies to Shiga toxins in a cohort of calves shedding Shiga-toxicogenic *Escherichia coli*. *Appl. Environ. Microbiol.* 75:3695–3704. doi:10.1128/AEM.02869-08
- Gething, M.J. 1999. Role and regulation of the ER chaperone BiP. *Semin. Cell Dev. Biol.* 10:465–472. doi:10.1006/scdb.1999.0318
- Gething, M.J., and J. Sambrook. 1992. Protein folding in the cell. *Nature*. 355:33–45. doi:10.1038/355033a0
- Gething, M.J., K. McCammon, and J. Sambrook. 1986. Expression of wild-type and mutant forms of influenza hemagglutinin: the role of folding in intracellular transport. *Cell*. 46:939–950. doi:10.1016/0092-8674(86)90076-0
- Goodnow, C.C., J. Crosbie, S. Adelstein, T.B. Lavoie, S.J. Smith-Gill, R.A. Brink, H. Pritchard-Briscoe, J.S. Wotherspoon, R.H. Loblay, K. Raphael, et al. 1988. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. *Nature*. 334:676–682. doi:10.1038/334676a0
- Greene, L.E., R. Zinner, S. Naficy, and E. Eisenberg. 1995. Effect of nucleotide on the binding of peptides to 70-kDa heat shock protein. *J. Biol. Chem.* 270:2967–2973. doi:10.1074/jbc.270.32.19022
- Haas, I.G., and T. Meo. 1988. cDNA cloning of the immunoglobulin heavy chain binding protein. *Proc. Natl. Acad. Sci. USA*. 85:2250–2254. doi:10.1073/pnas.85.7.2250
- Haas, I.G., and M. Wabl. 1983. Immunoglobulin heavy chain binding protein. *Nature*. 306:387–389. doi:10.1038/306387a0
- Hamman, B.D., L.M. Hendershot, and A.E. Johnson. 1998. BiP maintains the permeability barrier of the ER membrane by sealing the luminal end of the translocon pore before and early in translocation. *Cell*. 92:747–758. doi:10.1016/S0092-8674(00)81403-8
- Hayakawa, K., N. Hiramatsu, M. Okamura, J. Yao, A.W. Paton, J.C. Paton, and M. Kitamura. 2008. Blunted activation of NF-kappaB and NF-kappaB-dependent gene expression by geranylgeranylacetone: involvement of unfolded protein response. *Biochem. Biophys. Res. Commun.* 365:47–53. doi:10.1016/j.bbrc.2007.10.115
- Hu, C.C., S.K. Dougan, A.M. McGehee, J.C. Love, and H.L. Ploegh. 2009. XBP-1 regulates signal transduction, transcription factors and bone marrow colonization in B cells. *EMBO J.* 28:1624–1636. doi:10.1038/emboj.2009.117
- Jiang, J., K. Prasad, E.M. Lafer, and R. Sousa. 2005. Structural basis of interdomain communication in the Hsc70 chaperone. *Mol. Cell*. 20:513–524. doi:10.1016/j.molcel.2005.09.028
- Knittler, M.R., and I.G. Haas. 1992. Interaction of BiP with newly synthesized immunoglobulin light chain molecules: cycles of sequential binding and release. *EMBO J.* 11:1573–1581.
- Knittler, M.R., S. Dirks, and I.G. Haas. 1995. Molecular chaperones involved in protein degradation in the endoplasmic reticulum: quantitative interaction of the heat shock cognate protein BiP with partially folded immunoglobulin light chains that are degraded in the endoplasmic reticulum. *Proc. Natl. Acad. Sci. USA*. 92:1764–1768. doi:10.1073/pnas.92.5.1764
- Lass, A., M. Kujawa, E. McConnell, A.W. Paton, J.C. Paton, and C. Wójcik. 2008. Decreased ER-associated degradation of alpha-TCR induced by Grp78 depletion with the SubAB cytotoxin. *Int. J. Biochem. Cell Biol.* 40:2865–2879. doi:10.1016/j.biocel.2008.06.003
- Ma, Y., and L.M. Hendershot. 2001. The unfolding tale of the unfolded protein response. *Cell*. 107:827–830. doi:10.1016/S0092-8674(01)00623-7
- Matlack, K.E., B. Misselwitz, K. Plath, and T.A. Rapoport. 1999. BiP acts as a molecular ratchet during posttranslational transport of prepro-alpha factor across the ER membrane. *Cell*. 97:553–564. doi:10.1016/S0092-8674(00)80767-9
- Morinaga, N., K. Yahiro, G. Matsuura, J. Moss, and M. Noda. 2008. Subtilase cytotoxin, produced by Shiga-toxicogenic *Escherichia coli*, transiently inhibits protein synthesis of Vero cells via degradation of BiP and induces cell cycle arrest at G1 by downregulation of cyclin D1. *Cell. Microbiol.* 10:921–929. doi:10.1111/j.1462-5822.2007.01094.x
- Nataro, J.P., and J.B. Kaper. 1998. Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* 11:142–201.
- Paton, J.C., and A.W. Paton. 1998. Pathogenesis and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Clin. Microbiol. Rev.* 11:450–479.
- Paton, A.W., P. Srimanote, U.M. Talbot, H. Wang, and J.C. Paton. 2004. A new family of potent AB(5) cytotoxins produced by Shiga toxicogenic *Escherichia coli*. *J. Exp. Med.* 200:35–46. doi:10.1084/jem.20040392
- Paton, A.W., T. Beddoe, C.M. Thorpe, J.C. Whistock, M.C. Wilce, J. Rossjohn, U.M. Talbot, and J.C. Paton. 2006. AB5 subtilase cytotoxin inactivates the endoplasmic reticulum chaperone BiP. *Nature*. 443:548–552. doi:10.1038/nature05124
- Pelham, H.R. 1986. Speculations on the functions of the major heat shock and glucose-regulated proteins. *Cell*. 46:959–961. doi:10.1016/0092-8674(86)90693-8
- Reimold, A.M., N.N. Iwakoshi, J. Manis, P. Vallabhajosyula, E. Szomolanyi-Tsuda, E.M. Gravalles, D. Friend, M.J. Grusby, F. Alt, and L.H. Glimcher. 2001. Plasma cell differentiation requires the transcription factor XBP-1. *Nature*. 412:300–307. doi:10.1038/35085509
- Schmid, D., A. Baici, H. Gehring, and P. Christen. 1994. Kinetics of molecular chaperone action. *Science*. 263:971–973. doi:10.1126/science.8310296
- Shen, J., X. Chen, L. Hendershot, and R. Prywes. 2002. ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding

- and unmasking of Golgi localization signals. *Dev. Cell.* 3:99–111. doi:10.1016/S1534-5807(02)00203-4
- Skowronek, M.H., L.M. Hendershot, and I.G. Haas. 1998. The variable domain of nonassembled Ig light chains determines both their half-life and binding to the chaperone BiP. *Proc. Natl. Acad. Sci. USA.* 95:1574–1578. doi:10.1073/pnas.95.4.1574
- Takano, Y., N. Hiramatsu, M. Okamura, K. Hayakawa, T. Shimada, A. Kasai, M. Yokouchi, A. Shitamura, J. Yao, A.W. Paton, et al. 2007. Suppression of cytokine response by GATA inhibitor K-7174 via unfolded protein response. *Biochem. Biophys. Res. Commun.* 360:470–475. doi:10.1016/j.bbrc.2007.06.082
- Talbot, U.M., J.C. Paton, and A.W. Paton. 2005. Protective immunization of mice with an active-site mutant of subtilase cytotoxin of Shiga toxin-producing *Escherichia coli*. *Infect. Immun.* 73:4432–4436. doi:10.1128/IAI.73.7.4432–4436.2005
- Vanhove, M., Y.K. Usherwood, and L.M. Hendershot. 2001. Unassembled Ig heavy chains do not cycle from BiP in vivo but require light chains to trigger their release. *Immunity.* 15:105–114. doi:10.1016/S1074-7613(01)00163-7
- Voisine, C., E.A. Craig, N. Zufall, O. von Ahsen, N. Pfanner, and W. Voos. 1999. The protein import motor of mitochondria: unfolding and trapping of preproteins are distinct and separable functions of matrix Hsp70. *Cell.* 97:565–574. doi:10.1016/S0092-8674(00)80768-0
- Wang, H., J.C. Paton, and A.W. Paton. 2007. Pathologic changes in mice induced by subtilase cytotoxin, a potent new *Escherichia coli* AB5 toxin that targets the endoplasmic reticulum. *J. Infect. Dis.* 196:1093–1101. doi:10.1086/521364
- Wolfson, J.J., K.L. May, C.M. Thorpe, D.M. Jandhyala, J.C. Paton, and A.W. Paton. 2008. Subtilase cytotoxin activates PERK, IRE1 and ATF6 endoplasmic reticulum stress-signalling pathways. *Cell. Microbiol.* 10:1775–1786. doi:10.1111/j.1462-5822.2008.01164.x
- Yahiro, K., N. Morinaga, M. Satoh, G. Matsuura, T. Tomonaga, F. Nomura, J. Moss, and M. Noda. 2006. Identification and characterization of receptors for vacuolating activity of subtilase cytotoxin. *Mol. Microbiol.* 62:480–490. doi:10.1111/j.1365-2958.2006.05379.x
- Zhu, X., X. Zhao, W.F. Burkholder, A. Gragerov, C.M. Ogata, M.E. Gottesman, and W.A. Hendrickson. 1996. Structural analysis of substrate binding by the molecular chaperone DnaK. *Science.* 272:1606–1614. doi:10.1126/science.272.5268.1606
- Zou, Y.R., S. Takeda, and K. Rajewsky. 1993. Gene targeting in the Ig kappa locus: efficient generation of lambda chain-expressing B cells, independent of gene rearrangements in Ig kappa. *EMBO J.* 12:811–820.