The Schistosoma mansoni Protein Sm16/SmSLP/SmSPO-1 Assembles into a Nine-Subunit Oligomer with Potential To Inhibit Toll-Like Receptor Signaling

Kristoffer Brännström,1 Mikael E. Sellin,1 Per Holmfeldt,1 Maria Brattsand,2 and Martin Gullberg1*

Departments of Molecular Biology1 and Public Health and Clinical Medicine,2 Umeå University, Umeå, Sweden

Received 9 September 2008/Returned for modification 22 October 2008/Accepted 27 December 2008

Innate immunity provides a first line of defense toward invading microorganisms and depends on the expression of “pattern recognition receptors,” which recognize a restricted repertoire of molecules common to many pathogens (reviewed in reference 1). The Toll-like receptor (TLR) family is the archetypal pattern recognition receptor type and triggers signaling pathways leading to inflammatory responses through the production of an array of cytokines/chemokines. TLR signaling involves a variety of adaptor proteins, e.g., the bridging adaptor MAL and MyD88, which recruit members of the interleukin-1 receptor-associated kinase (IRAK) family to specific TLRs (reviewed in reference 1). While most of the identified TLR ligands are of viral, bacterial, or fungal origin, recent reports suggest that components of parasitic helminths may also act as TLR ligands (reviewed in reference 1). The Toll-like receptor (TLR) family is the archetypal pattern recognition receptor type and triggers signaling pathways leading to inflammatory responses through the production of an array of cytokines/chemokines. TLR signaling involves a variety of adaptor proteins, e.g., the bridging adaptor MAL and MyD88, which recruit members of the interleukin-1 receptor-associated kinase (IRAK) family to specific TLRs (reviewed in reference 1). While most of the identified TLR ligands are of viral, bacterial, or fungal origin, recent reports suggest that components of parasitic helminths may also act as TLR ligands (reviewed in reference 1). The life cycle of the helminth Schistosoma mansoni, the parasite that causes schistosomiasis, involves human and snail hosts, as well as two free-living waterborne larval stages (reviewed in reference 4). The developmental stages include the miracidium that is infectious to fresh water snails, the sporocyst that is the parasitic form in the snail, and the cercaria that is released by snails and penetrates the skin of the human host. During entry into the dermis, the parasite transforms into a schistosomulum. The parasite then enters the blood system and develops into the adult worm, which is the long-term parasitic form.
the cytosol by penetration of lipid bilayers. It follows that the physiological function of the cell surface-binding Sm16 protein seems likely to involve interactions on the extracellular and/or luminal side of membranes of host cells.

In the present study, we performed structural and functional studies of the secreted form of Sm16 engineered to allow recombinant expression of a soluble protein in either Escherichia coli or, to circumvent pyrogen/endotoxin contamination, the methylotrophic yeast Pichia pastoris. We show that Sm16 forms an approximately nine-subunit oligomer that has the potential to inhibit TLR signaling of monocytes without detectable inhibitory/toxic effects on T-lymphocyte activation or proliferation of human cells. Our findings suggest a TLR-dependent mechanism whereby Sm16 secretion from the acetal-bular glands may inhibit activation of the innate immune response during skin penetration.

MATERIALS AND METHODS

DNA constructs. Low expression of Sm16 from the native cDNA (18), which in part can be attributed to many rare codons for mammals and E. coli, prompted us to construct a codon-optimized synthetic Sm16-encoding gene by ligation of a set of overlapping oligonucleotides. This synthetic sequence encodes amino acid 23 to 117 of Sm16 [termed Sm16(23-117), which corresponds to the secreted form of Sm16 (9)]. An NcoI site was introduced at the ATG initiation codon, and a SacI site was introduced by silent mutations within the codons for the last three C-terminal amino acids (Glu-Ser-Ser) (9). To construct the engineered version of Sm16 that contains Ala substitutions at the codons for Ile-92 and Leu-93, termed Sm16(23-117)AA, we employed a PCR strategy using the synthetic Sm16-encoding gene as a template and 3′ primers specifying the substitutions, as well as a C-terminal DYKDDDDK Flag epitope tag (primer sequences are available on request). To construct stepwise C-terminal deletions (outlined in Fig. 1A; primer sequences are available on request), we employed a PCR-based strategy using 3′ primers defining each one of the deletions. These primers all contained a SacI site within the sequence that corresponds to the last three codons of the Sm16 sequence. The PCR products were then used to replace the NcoI-SacI fragment of Flag epitope-tagged Sm16(23-117)AA. For expression in E. coli, the coding sequences of Flag-tagged Sm16 derivatives were subcloned as NcoI-SacI fragment of Flag epitope-tagged Sm16(23-117)AA, with improved solubility and protein expression. (A) Sche-

Expression of Sm16 in E. coli and P. pastoris, purification of recombinant proteins, and detection of endotoxins and pyrogenic activities. Sm16 proteins expressed in E. coli and P. pastoris contained an N-terminal six-His tag and an eight-residue C-terminal Flag tag. T7 expression plasmid pET3d was transformed into E. coli strain BL21(DE3)pLysS, and recombinant His-tagged Sm16 proteins were purified by metal ion affinity as described previously (19), with the modifications that all of the buffers contained 5 mM 2-mercaptoethanol and that HisTrap crude columns (Amersham Pharmacia Biotech) were subjected to an extensive washing protocol described above for Sm16 expressed in E. coli and disrupted with a bead beater. Lysates were cleared by centrifugation (100,000 × g for 1 h) and filtered through a 0.22-μm filter. The His-tagged Sm16 proteins were then purified by two rounds of metal ion affinity purification by the ABI PRISM dye terminator cycle sequenc-


The coding sequences of PCR-generated fragments were confirmed by nucleotide sequence analysis with an ABI PRISM dye terminator cycle sequenc-

For the detection of endotoxins by the Limulus amoebozyte lysate test, a QCL-1000 turbidometric chromogenic kit (Lonza) was used according to the manufacturer’s instructions; the detection limit of the test was <0.1 endotoxin

![FIG. 1. Construction of the engineered version of Sm16, Sm16(23-117)AA, with improved solubility and protein expression. (A) Schematic representation of recombinant Sm16 derivatives with successive truncations at the C terminus. Each truncated derivative is defined according to the amino acid sequence deduced from the cDNA sequence (numbers in parentheses). The location of hydrophobic residues Ile-92 and Leu-93 is indicated by a dashed line. The signal peptide of Sm16 consists of the first 22 residues, which are absent in the secreted form of the protein termed Sm16(23-117). All Sm16 deriva-

FIG. 1. Construction of the engineered version of Sm16, Sm16(23-117)AA, with improved solubility and protein expression. (A) Schematic representation of recombinant Sm16 derivatives with successive truncations at the C terminus. Each truncated derivative is defined according to the amino acid sequence deduced from the cDNA sequence (numbers in parentheses). The location of hydrophobic residues Ile-92 and Leu-93 is indicated by a dashed line. The signal peptide of Sm16 consists of the first 22 residues, which are absent in the secreted form of the protein termed Sm16(23-117). All Sm16 derivatives contain an N-terminal His tag and an eight-residue C-terminal Flag tag. (B) Anti-Sm16 antibodies were used to detect recombinant Sm16 by immunoblot analysis of crude lysates of E. coli transformed with pET3d plasmid derivatives directing the expression of the indicated Sm16 proteins. (C) Coomassie brilliant blue-stained SDS-PAGE (upper panel) and immunoblot analysis with anti-Sm16 (lower panel) of crude lysates of E. coli transformed with pET3d derivatives directing the expression of the indicated Sm16 proteins. The abundantly expressed Sm16(23-117)AA protein was modified for improved solubility by replacing Ile-92 and Leu-93 with Ala, which also greatly improved expression. (D) Far-UV circular dichroism (CD) spectra of Sm16(23-117)AA expressed in E. coli as a His-tagged protein and purified by metal ion affinity. Proteins were dissolved at 0.2 mg/ml in 2 mM sodium phosphate buffer and scanned at 20°C. The data indicate that ~70% of the protein has an α-helical conformation. The values to the left of panels B and C are molecular sizes in kilodaltons.
Sn16 antibodies, detection of cell surface binding of Sn16, flow cytometry, and immunoblotting. Antibodies to Sn16 were raised by immunizing rabbits with a His-tagged Sn16-(23-117)-nonrelated helix fusion protein expressed by and purified from E. coli (9). The resulting antibodies were affinity purified by absorption to Sm16(23-70) coupled to Sepharose, which resulted in antibodies that allowed equivalent detection of full-length and C-terminally truncated Sm16 proteins. For analysis of Sn16 binding to cell surfaces, human K562 erythroleukemia cells were incubated (1-10^5 in 0.5 ml RPMI 1640 medium-10% fetal calf serum [FCS]) with 2 μg/ml Sn16 protein for 30 min at 37°C. Cells were subsequently washed two times in phosphate-buffered saline, and surface-bound Sn16 was detected by staining of live cells with affinity-purified anti-Sn16 (1 μg/ml) and fluorescent-conjugated anti-rabbit immunoglobulin. Cell surface fluorescence was quantitated by flow cytometric analysis of live cells (>95% of the cells were viable and included in the acquisition gate, and >200,000 cells were collected) with a FACSCalibur flow cytometer together with CellQuest software (BDB Biosciences). For analysis of TLR4 cell surface expression levels, live cells were stained with Alexa 488-conjugated anti-CD80 (B-5-1-2; AbD Serotec) and fluorescence intensity was determined by flow cytometry. Immunoblotting and subsequent detection of the ECL detection system (Amersham Pharmacia Biotech) were performed with anti-α-tubulin (B-5-1-2, Sigma), Isb-α (C-15, sc203; Santa Cruz Biotechnology), and affinity-purified rabbit anti-Sn16 and anti-IRAK1 (H-273; Santa Cruz Biotechnology, Inc.) as described previously (10). For quantitative analysis of immunoblotts, the Bio-Rad ChemDoc (Bio-Rad) system was used with the Quantity One 4.4 program.

Circular-dichroism spectra and determinations of hydrodynamic parameters and Stokes radii of Sn16 were acquired by using a Jasco Yvon CD6 spectropolarimeter. The spectra between 190 and 260 nm were recorded by collecting data at 1-nm intervals with an integration time of 2 s (scan speed of 0.5 nm s^-1). The Stokes radius was estimated by gel filtration chromatography on a Hi Load 16/60 Superdex 75 column with the AKTA purifier fast protein liquid chromatography system (Amersham Pharmacia Biotech). The elution buffer was phosphate-buffered saline, and columns were calibrated with standard proteins with known Stokes radii (cytochrome c, 1.70 nm; catalase, 11.3S). Twenty fractions (cytochrome c, 1.70 nm; catalase, 11.3S). Twenty fractions (1 ml each) were collected, and aliquots of 1 ml each were assayed for Sn16 expression and aggregation of the purified protein under physiological buffer conditions, which obstructed studies requiring a pure and soluble Sn16 protein. To overcome these problems, the Sn16 polypeptide was scanned for aggregation-promoting regions that may interfere with expression and solubility. Analysis of a series of C-terminally truncated derivatives indicated that poor Sn16 protein expression depends entirely on the short sequence Lys^{98}-Ile-Leu-Gly^{94}, as defined by the Sn16(23-94) and Sm16(23-90) proteins (Fig. 1B). We also found that a purified preparation of the highly expressed Sm16(23-90) protein remained soluble in physiological buffers (data not shown).

We have previously studied Sm16 secretion by transfection of cell lines and confirmed cleavage of the signal peptide between amino acids 22 and 23 during passage through the secretory pathway (9). Using E. coli to express the 95-residue secreted form of Sm16, termed Sm16(23-117) (Fig. 1A), we noted problems with poor expression and aggregation of the purified protein under physiological buffer conditions, which obstructed studies requiring a pure and soluble Sm16 protein. To overcome these problems, the Sn16 polypeptide was scanned for aggregation-promoting regions that may interfere with expression and solubility. Analysis of a series of C-terminally truncated derivatives indicated that poor Sn16 protein expression depends entirely on the short sequence Lys^{98}-Ile-Leu-Gly^{94}, as defined by the Sm16(23-94) and Sm16(23-90) proteins (Fig. 1B). We also found that a purified preparation of the highly expressed Sm16(23-90) protein remained soluble in physiological buffers (data not shown).

As a strategy to decrease the aggregation propensity of Sm16, the hydrophobicity within the identified region was decreased by replacing Ile-92 and Leu-93 with Ala residues; these substitutions are indicated by the AA superscript in the modified Sm16(23-117)^AA protein name. As shown in Fig. 1C, immunoblot analysis of bacterial lysates shows that both of these epitope-tagged recombinant proteins migrate at ~20 kDa but that Sm16(23-117)^AA was expressed at 24-fold higher levels than the unmodified Sm16(23-117) protein.

While the pure unmodified Sm16(23-117) protein was soluble in 0.5 M imidazole-0.5 M NaCl, pH 8.0, it formed visible aggregates within 1 h in phosphate-buffered saline. However, the modified Sm16(23-117)^AA protein was readily soluble in phosphate-buffered saline and purified preparations could be stored for prolonged periods without aggregating (data not shown). Thus, the present modification solved all of the problems we experienced with aggregation and consequent precipitation, which was a prerequisite for the present biochemical and functional characterization of a purified soluble Sm16 protein.

The amino acid composition and sequence of Sm16 suggest a high propensity to form an α-helical secondary structure. This propensity was experimentally confirmed by measurement of the far-UV circular-dichroism spectra, which revealed that ~70% of the polypeptide exists in an α-helical configuration at 20°C (Fig. 1D).
Sm16 is an approximately nine-subunit oligomer. When analyzed by SDS-PAGE, Sm16(23-117)AA migrates at ~19 kDa under reducing conditions (Fig. 2A), which is slower than the deduced 13.6-kDa molecular mass of this epitope-tagged recombinant protein. However, by immunoblotting of cercarial extract, we could confirm that the native Sm16 protein also migrated slowly on SDS-PAGE compared with its deduced molecular mass of 11.3 kDa (Fig. 2D).

To characterize the engineered version of secreted Sm16 and to compare it with native Sm16 from cercarial extract, we employed a combination of gel filtration chromatography and sucrose gradient sedimentation. This provides estimates of the Stokes radius and the sedimentation coefficient (S), which allows calculation of the molecular masses of native complexes with the formula given in Materials and Methods. As shown in Fig. 2B and C, analysis of purified Sm16(23-117)AA indicates a 5.5-nm Stokes radius and a 5.4S sedimentation coefficient, which reveals that the protein forms an oligomer with an estimated molecular mass of ~122 kDa. Importantly, parallel analysis of cercarial extracts (Fig. 2E and F), combined with immunodetection of Sm16 in fractions, revealed hydrodynamic parameters similar to those of the pure Sm16(23-117)AA protein (Stokes radii and sedimentation coefficients are indicated in the panels of Fig. 2).

The combined evidence from analysis of purified Sm16(23-117)AA and native Sm16 in crude cercarial extracts indicates that Sm16 is an oligomeric protein with a defined number of subunits. Based on the deduced 13.6-kDa molecular mass of Sm16(23-117)AA, an estimated molecular mass of ~122 kDa of the oligomer, and the ~10% uncertainty of the method (6), it can be estimated that Sm16 is a 9 ± 1-subunit oligomer.

Characterization of a biochemically distinct Sm16 trimer in a preparation of C-terminally truncated Sm16 protein. To evaluate the importance of the C terminus for the oligomeric state of Sm16, a truncated derivative was analyzed by gel filtration. Interestingly, the purified Sm16(23-98)AA protein, which lacks the C-terminal 19 residues, was resolved into two distinct peaks on a Sephadex 200 column (Fig. 3A; SDS-PAGE analysis of pooled peak fractions is shown as an insert). Pooled peak fractions of the 5.5-nm species and the 3.1-nm species were further analyzed by sucrose gradient sedimentation (Fig. 3B and C). By this strategy, the Stokes radius and sedimentation coefficient were estimated for each of the two oligomeric forms of the C-terminally truncated Sm16(23-98)AA derivative. The data indicate that the Sm16(23-98)AA protein exists in two stable oligomeric forms which, based on their hydrodynamic properties, have estimated molecular masses of ~92 kDa (5.5 nm and 4.0S) and ~29 kDa (3.1 nm and 2.2S). Given the deduced 11.2-kDa molecular mass of Sm16(23-98)AA and the general ~10% uncertainty of the method, the molecular mass of the large oligomer is consistent with a 9-mer, which implies the same oligomeric state as the full-length Sm16(23-117)AA protein. Moreover, the estimated 29-kDa molecular mass of the small oligomer indicates that a large proportion of the expressed Sm16(23-98)AA proteins also form stable approximately three-subunit oligomers. This result reveals a propensity of Sm16 to also form stable trimers, which may be relevant...
for the structure of the approximately nine-subunit native oligomer.

Previous studies have shown that cell surface binding of the secreted form of full-length Sm16 is not inhibited by the polyanionic substance heparin (9). Consistent with this result, the approximately nine-subunit oligomer of Sm16(23-98)AA with a 5.5-nm Stokes radius was found not to bind immobilized heparin at a physiological salt concentration (i.e., 0.15 M NaCl, Fig. 3D). Interestingly, however, the approximately three-subunit oligomer with a 3.1-nm Stokes radius bound avidly to heparin and was eluted at ~0.5 M NaCl (Fig. 3E). Hence, the approximately three-subunit oligomer exposes polyanion binding surfaces that appear to be hidden in the native approximately nine-subunit Sm16 oligomer, which indicates a differential polypeptide arrangement within these two oligomeric forms. The presence of these two oligomeric forms may be interpreted to suggest that Sm16 is a nine-subunit trimer of trimers.

The C-terminal part of Sm16 is required for both oligomerization and binding to the surface of human cells. Analysis of a series of truncated engineered versions of Sm16 proteins by Sephadex 200 gel filtration shows that consecutive C-terminal truncations of Sm16 result in a stepwise decrease in the fraction of approximately nine-subunit oligomers (Fig. 4A). Consistent with the analysis of Sm16(23-98)AA (Fig. 3), we observed apparent trimers in these preparations of truncated Sm16 and the proportion of trimers depended on the extent of the truncation (data not shown). Thus, it appears that multiple physically separated C-terminal regions of Sm16 contribute to the assembly of the approximately nine-subunit oligomer.

We have previously found that Sm16 binds both to protein-free liposomes and to the surfaces of various human cell lines. Binding does not involve polyanion interactions with cell surface proteoglycans, as evidenced by a lack of inhibition by heparin (9), which indicates that Sm16 binds to the cell surface through interaction with the lipid bilayer of the plasma membrane. To analyze the role of C-terminal regions in cell surface binding of the approximately nine-subunit oligomer, Sephadex 200 gel filtration was used to isolate the fraction of large oligomers of the relevant Sm16 proteins [i.e., all derivatives except Sm16(23-70)]. This ensured that this analysis of cell binding is unbiased by a differential oligomerization propensity, and it is notable that the isolated oligomers in all cases appeared stable, as confirmed by a second round of gel filtration (data not shown). Cell surface binding was determined by incubation with human K562 cells, followed by detection of cell surface-associated Sm16 with specific antibodies and flow cytometry. The data revealed that consecutive C-terminal truncations of Sm16 result in a stepwise decrease in cell surface binding and that truncation of 27 residues severely reduced binding (Fig. 4B). Thus, multiple small regions of the C-terminal part of Sm16 contribute to cell surface binding independently of their importance for oligomerization.

Biological activities of Sm16 on blood cells. Interpretations of immunomodulatory activities exerted by a bacterially expressed lipid-binding protein is likely to be confounded by both formulation of the N-terminal methionine and trace amounts of endotoxin/pyrogen contamination. We therefore used the methylotrophic yeast P. pastoris for the expression of Sm16(23-117)AA-P (the letter P denotes Pichia) and developed a stringent washing protocol during purification to eliminate pyrogenic contaminants (see Materials and Methods). The purified Sm16(23-117)AA-P protein was found to be indistinguishable from the E. coli-derived protein with respect to the Stokes radius, migration on SDS-PAGE, and binding to the surfaces of human cells (data not shown).
Anti-CD3 antibodies activate T lymphocytes through the CD3/antigen receptor complex, which induces T-lymphocyte proliferation by a mechanism that depends on collaboration with monocytic cells (2). This collaboration induces cytokine production by monocytic cells, e.g., IL-1β, which is required for subsequent production of the lymphokine IL-2 by T lymphocytes. To evaluate potential immunosuppressive activities of Sm16(23-117)AA, the effects on isolated blood mononuclear cells were analyzed in the presence or absence of CD3 stimulation. As shown in Fig. 5A and B, a high concentration of Sm16(23-117)AA (50 μg/ml) had no effect by itself and did not inhibit anti-CD3 antibody-stimulated IL-1β or IL-2 expression. Moreover, we did not detect inhibition of DNA synthesis and the basal proliferation actually appeared to be somewhat increased in the presence of Sm16(23-117)AA (Fig. 5C). Thus, we found no evidence that Sm16(23-117)AA exerts general immunomodulatory activities.

To search for Sm16-dependent effects on innate immunity, we analyzed the cytokine production of human blood in the absence or presence of the bacterial product lipopolysaccharide (LPS). As expected, Sm16(23-117)AA alone did not stimulate cytokine production (Fig. 6, open squares). Interestingly, however, the full-length Sm16(23-117)AA protein potently suppressed LPS-induced production of IL-6, tumor necrosis factor alpha (TNF-α), and IL-1β and 50% inhibition was observed at around 2 μg/ml in all cases (Fig. 6, filled squares). As a control, we also studied the effect of the C-terminally truncated Sm16(23-70)P protein, which does not bind to cell surfaces (Fig. 4B). As expected, this extensively truncated Sm16 protein did not inhibit the LPS response (Fig. 6, filled triangles). It should be noted that the dose response of Sm16(23-117)AA-mediated inhibition was similar over a broad range of LPS concentrations (1 to 100 ng/ml; data not shown), which indicates that Sm16 does not inhibit by sequestering of LPS or by competing for TLR4 binding.

Sm16 inhibits cytokine production in response to structurally distinct TLR ligands. As outlined above, IL-1β production during an anti-CD3 antibody-induced mitogenic response depends on T-lymphocyte-dependent stimulation of monocytes (reviewed in reference 2). Thus, our finding that Sm16(23-117)AA does not inhibit IL-1β expression in response to anti-CD3 antibodies (Fig. 5A) while LPS-induced production of IL-6, TNF-α, and IL-1β is potently suppressed (Fig. 6) indicates that Sm16 does not exert a general inhibition of monocyte cytokine production. To further characterize inhibition of cytokine production by Sm16(23-117)AA, human blood was stimulated with saturating concentrations of the TLR4 ligand LPS, the synthetic TLR3 ligand poly(I:C), and the TLR2 ligand poly(I:C).

Subsequent production of the lymphokine IL-2 by T lymphocytes. To evaluate potential immunosuppressive activities of Sm16(23-117)AA, the effects on isolated blood mononuclear cells were analyzed in the presence or absence of CD3 stimulation. As shown in Fig. 5A and B, a high concentration of Sm16(23-117)AA (50 μg/ml) had no effect by itself and did not inhibit anti-CD3 antibody-stimulated IL-1β or IL-2 expression. Moreover, we did not detect inhibition of DNA synthesis and the basal proliferation actually appeared to be somewhat increased in the presence of Sm16(23-117)AA (Fig. 5C). Thus, we found no evidence that Sm16(23-117)AA exerts general immunomodulatory activities.

To search for Sm16-dependent effects on innate immunity, we analyzed the cytokine production of human blood in the absence or presence of the bacterial product lipopolysaccharide (LPS). As expected, Sm16(23-117)AA alone did not stimulate cytokine production (Fig. 6, open squares). Interestingly, however, the full-length Sm16(23-117)AA protein potently suppressed LPS-induced production of IL-6, tumor necrosis factor alpha (TNF-α), and IL-1β and 50% inhibition was observed at around 2 μg/ml in all cases (Fig. 6, filled squares). As a control, we also studied the effect of the C-terminally truncated Sm16(23-70)P protein, which does not bind to cell surfaces (Fig. 4B). As expected, this extensively truncated Sm16 protein did not inhibit the LPS response (Fig. 6, filled triangles). It should be noted that the dose response of Sm16(23-117)AA-mediated inhibition was similar over a broad range of LPS concentrations (1 to 100 ng/ml; data not shown), which indicates that Sm16 does not inhibit by sequestering of LPS or by competing for TLR4 binding.

Sm16 inhibits cytokine production in response to structurally distinct TLR ligands. As outlined above, IL-1β production during an anti-CD3 antibody-induced mitogenic response depends on T-lymphocyte-dependent stimulation of monocytes (reviewed in reference 2). Thus, our finding that Sm16(23-117)AA does not inhibit IL-1β expression in response to anti-CD3 antibodies (Fig. 5A) while LPS-induced production of IL-6, TNF-α, and IL-1β is potently suppressed (Fig. 6) indicates that Sm16 does not exert a general inhibition of monocyte cytokine production. To further characterize inhibition of cytokine production by Sm16(23-117)AA, human blood was stimulated with saturating concentrations of the TLR4 ligand LPS, the synthetic TLR3 ligand poly(I:C), and the TLR2 ligand poly(I:C).
ligands peptidoglycan (PG) and synthetic lipopeptide Pam3CSK4. The data reveal efficient Sm16(23-117)AA
mediated inhibition of the response to either LPS or poly(I:C), while the responses to PG and Pam3CSK4 were only partially inhibited (Fig. 7, upper panels). Thus, Sm16(23-117)AA
inhibits the responses to several types of TLR ligands but with large differences in the apparent efficiency.

Sm16 has previously been described as an IL-1 receptor antagonist (IL-1RA)-inducing protein (16). However, analysis of the anti-inflammatory cytokine IL-1RA revealed only a marginal increase in the basal level in the presence of Sm16(23-117)AA
(Fig. 7, lower panels). Thus, Sm16(23-117)AA
inhibits the responses to several types of TLR ligands but with large differences in the apparent efficiency.

Sm16 has previously been described as an IL-1 receptor antagonist (IL-1RA)-inducing protein (16). However, analysis of the anti-inflammatory cytokine IL-1RA revealed only a marginal increase in the basal level in the presence of Sm16(23-117)AA
(Fig. 7, lower panels). Thus, Sm16(23-117)AA
inhibits the responses to several types of TLR ligands but with large differences in the apparent efficiency.

Sm16 has previously been described as an IL-1 receptor antagonist (IL-1RA)-inducing protein (16). However, analysis of the anti-inflammatory cytokine IL-1RA revealed only a marginal increase in the basal level in the presence of Sm16(23-117)AA
(Fig. 7, lower panels). Thus, Sm16(23-117)AA
inhibits the responses to several types of TLR ligands but with large differences in the apparent efficiency.

FIG. 6. Effect of P. pastoris-expressed Sm16 proteins on LPS-induced production of proinflammatory cytokines in whole blood. Human blood was preincubated for 10 min with graded concentrations of P. pastoris-expressed Sm16(23-70)P (triangles) or Sm16(23-117)AA
(squares). Cells were then cultured for 6 h in the absence (open symbols) or presence (filled symbols) of LPS (50 ng/ml), followed by analysis of IL-6 (A), TNF-α (B), and IL-1β (C) in the supernatants. The data plotted are representative of at least 10 independent analyses performed in triplicate. Blood cells from four healthy donors were analyzed and found to be indistinguishable with regard to the effect of purified Sm16 derivatives.

FIG. 7. Effect of Sm16(23-117)AA
on cytokine production in response to various TLR ligands. Human blood was preincubated for 10 min with a buffer control (open bars) or P. pastoris-expressed Sm16(23-117)AA
(20 μg/ml) (filled bars). The buffer control, LPS (50 ng/ml), poly(I:C) (20 μg/ml), PG (5 μg/ml), or Pam3CSK4 (5 μg/ml) was then added as indicated for 6 h of incubation, followed by analysis of either IL-6 (upper panel) or IL-1RA (lower panels) in the supernatants. The data plotted are representative of at least four independent analyses performed in triplicate with blood from three donors.

IL-1RA secretion in response to either of these two TLR2 ligands (Fig. 7).

The finding that Sm16(23-117)AA
significantly inhibits both LPS- and poly(I:C)-induced IL-1RA secretion, as well as very potently inhibits the IL-6 response to these distinct TLR ligands, suggests that Sm16 has the potential to interfere with TLR signaling. While we cannot explain why the PG and Pam3CSK4 response is poorly inhibited by Sm16, these results still illustrate the specificity of Sm16-mediated inhibition of the LPS and poly(I:C) response.

Sm16 inhibits a TLR-proximal signaling event. To address the mechanism behind the inhibitory action of Sm16, we used a clonal human monocytic leukemia cell line, Mono-Mac-6, as a model system. These monocytic cells respond to TLR stimulation by induction of cytokine expression and by maturation to macrophages, which is associated with decreased cell proliferation (25). As shown in Fig. 8A, Sm16(23-117)AA
alone has no detectable effect on the proliferation of Mono-Mac-6. Significantly, however, the presence of Sm16(23-117)AA
partially counteracts the decrease in cell proliferation observed after 4 days of stimulation with 2 ng/ml LPS. Moreover, flow cytometry did not indicate that Sm16 modulates the surface expression of the LPS-binding TLR4 complex (Fig. 8B). These results establish that Sm16(23-117)AA
does not cause any

FIG. 8. Effect of Sm16(23-117)AA
on the clonal monocytic cell line Mono-Mac-6. (A) Mono-Mac-6 cells seeded at 0.5 × 10⁶/ml and cultured for 4 days with either a buffer control or LPS (2 ng/ml), as indicated, in the absence (open bars) or presence (filled bars) of Sm16(23-117)AA
(20 μg/ml). Cell proliferation was analyzed by determination of [³H]thymidine uptake during the last 24 h of culture. (B) Mono-Mac-6 cells were cultured for 5 h in the absence (open bars) or presence (filled bars) of Sm16(23-117)AA
(20 μg/ml). TLR4 surface expression was then analyzed by flow cytometry and expressed as the mean fluorescence signal.
general inhibitory/cytotoxic effects on Mono-Mac-6 cells and reveal counteraction of LPS-induced cell proliferation inhibition without detectable effects on TLR4 surface expression.

To further characterize the action of Sm16(23-117) \( ^{\Delta \Delta P} \) on Mono-Mac-6 cells, we analyzed the production of the proinflammatory cytokine IL-6 in response to LPS, poly(I:C), and PG. As shown in Fig. 9, top panels, while the response to LPS and poly(I:C) was efficiently blocked, the response to PG was only partially inhibited. We also observed similar inhibition by analysis of TNF-\( \alpha \) and IL-1B (data not shown).

We also analyzed the production of the two anti-inflammatory cytokines IL-1RA and IL-10 and found a partial inhibition by Sm16(23-117) \( ^{\Delta \Delta P} \) in the presence of either LPS or poly(I:C) (Fig. 9). It was also evident that the production of both IL-1RA and IL-10 is essentially unaltered in PG-stimulated Mono-Mac-6 cells. Thus, Sm16 appears to have similar actions on TLR ligand-induced responses of a clonal monocytic cell line and whole blood cells (compare Fig. 7 and 9).

The family of NF\( \kappa B \) transcription factors is central for TLR-mediated signaling events. Mono-Mac-6 cells were preincubated for 10 min in the absence or presence of Sm16(23-117) \( ^{\Delta \Delta P} \) (20 \( \mu \)g/ml) as indicated. A buffer control (Co), LPS (50 ng/ml), or PG (5 \( \mu \)g/ml) was then added as indicated. Cells were incubated at 37°C for 0.5 h (A), 1 h (B, top), or 8 h (B, bottom) and processed for immunoblot analysis. Filters were probed with either I\( \kappa B \)-\( \alpha \) (A) or anti-IRAK1 (B). Anti-\( \alpha \)-tubulin was used as a control for equal loading. The presented data are representative of at least three independent analyses.

**FIG. 9.** Effect of Sm16(23-117) \( ^{\Delta \Delta P} \) on cytokine production by Mono-Mac-6 cells in response to various TLR ligands. Mono-Mac-6 cells were preincubated for 10 min with a buffer control (open bars) or 20 \( \mu \)g/ml Sm16(23-117) \( ^{\Delta \Delta P} \) (filled bars). The buffer control, LPS (50 ng/ml), poly(I:C) (20 \( \mu \)g/ml), or PG (5 \( \mu \)g/ml) was then added as indicated for 8 h of incubation, followed by quantification of the proinflammatory cytokine IL-6 or the anti-inflammatory cytokines IL-1RA and IL-10 in the supernatants. The data plotted are representative of at least four independent analyses performed in triplicate.

Inhibition by analysis of TNF-\( \alpha \) and IL-1B (data not shown).

We also analyzed the production of the two anti-inflammatory cytokines IL-1RA and IL-10 and found a partial inhibition by Sm16(23-117) \( ^{\Delta \Delta P} \) in the presence of either LPS or poly(I:C) (Fig. 9). It was also evident that the production of both IL-1RA and IL-10 is essentially unaltered in PG-stimulated Mono-Mac-6 cells. Thus, Sm16 appears to have similar actions on TLR ligand-induced responses of a clonal monocytic cell line and whole blood cells (compare Fig. 7 and 9).

The family of NF\( \kappa B \) transcription factors is central for TLR-mediated signaling events. Mono-Mac-6 cells were preincubated for 10 min in the absence or presence of Sm16(23-117) \( ^{\Delta \Delta P} \) (20 \( \mu \)g/ml) as indicated. A buffer control (Co), LPS (50 ng/ml), or PG (5 \( \mu \)g/ml) was then added as indicated. Cells were incubated at 37°C for 0.5 h (A), 1 h (B, top), or 8 h (B, bottom) and processed for immunoblot analysis. Filters were probed with either I\( \kappa B \)-\( \alpha \) (A) or anti-IRAK1 (B). Anti-\( \alpha \)-tubulin was used as a control for equal loading. The presented data are representative of at least three independent analyses.

**FIG. 10.** Effect of Sm16(23-117) \( ^{\Delta \Delta P} \) on TLR ligand-induced intracellular signaling events. Mono-Mac-6 cells were preincubated for 10 min in the presence of Sm16(23-117) \( ^{\Delta \Delta P} \) (20 \( \mu \)g/ml) as indicated. A buffer control (Co), LPS (50 ng/ml), or PG (5 \( \mu \)g/ml) was then added as indicated. Cells were incubated at 37°C for 0.5 h (A), 1 h (B, top), or 8 h (B, bottom) and processed for immunoblot analysis. Filters were probed with either I\( \kappa B \)-\( \alpha \) (A) or anti-IRAK1 (B). Anti-\( \alpha \)-tubulin was used as a control for equal loading. The presented data are representative of at least three independent analyses.

Activation of TLR2 or TLR4 triggers binding of the MyD88 adaptor to the intracellular portion of the receptors via the bridging adaptor MAL. This initiates a signaling cascade that involves recruitment of IRAK4 and IRAK1 proximal to the TLR complex and subsequent phosphorylation and degradation of IRAK1 (1). Accordingly, to evaluate whether Sm16 inhibits TLR signaling proximal to the receptor complex, we analyzed LPS- and PG-mediated IRAK1 degradation in the absence or presence of Sm16(23-117) \( ^{\Delta \Delta P} \). As shown by immunoblotting in Fig. 10B, LPS induced a persistent reduction of IRAK1 protein content that was efficiently inhibited by Sm16(23-117) \( ^{\Delta \Delta P} \). Significantly, however, the presence of Sm16(23-117) \( ^{\Delta \Delta P} \) did not significantly inhibit IRAK1 degradation in PG-stimulated Mono-Mac-6 cells, which seems consistent with the notion that PG-stimulated cytokine secretion is only partially inhibited under the same conditions (Fig. 9). These results confirm the specificity of the observed Sm16(23-117) \( ^{\Delta \Delta P} \)-mediated inhibition of IRAK1 degradation in LPS-stimulated cells.

The secreted Sm16 protein binds to the cell surface but lacks apparent cell membrane-penetrating properties (9), which makes it unlikely that the mechanism behind Sm16-mediated inhibition of TLR signaling involves direct interactions with cytosolic signaling proteins. Thus, it seems more likely that Sm16 exert its inhibitory action by interfering with ligand activation of the TLR complex. This implies that Sm16 acts upstream of the TLR-proximal IRAK1 protein, which is indeed shown in Fig. 10B.
DISCUSSION

Sm16 has been described as a stathmin-like protein (SmSLP), which implies similarity to a cytosolic microtubule regulator in animal cells (23). The evidence includes ~26% identity of amino acid sequences and reported tubulin-binding and microtubule-destabilizing activities of a partially purified Sm16 preparation. However, by comparing recombinant Sm16 and stathmin proteins, we have reported a failure to reproduce stathmin-like activities of Sm16 and also found that stathmin does not share the demonstrated lipid bilayer-binding properties of Sm16. In addition, to introduce Sm16 into the cytosol of human cells, i.e., the same location as stathmin, we expressed a signal peptide-deficient Sm16 derivative, which was found to cause apoptotic cell death without evidence of stathmin-like microtubule regulatory activity (9). While these results illustrate a striking functional difference between Sm16 and stathmin, the proapoptotic effect of cytosolic Sm16 probably lacks a physiological significance since the secreted Sm16 protein evidently lacks cell membrane-penetrating properties (9). Both the Sm16 and stathmin sequences have characteristics indicating a high degree of α-helical secondary structure, which has also been experimentally confirmed (Fig. 1D and reference 24), but in the present study we demonstrate that Sm16 is an oligomeric protein, in contrast to the monomeric structure of stathmin (7, 22). Thus, Sm16 and stathmin are clearly very different with respect to both function and structure.

The present analysis of truncated Sm16 protein derivatives allowed us to design an engineered version of the secreted form of Sm16 with a decreased aggregation propensity and increased expression in E. coli (Fig. 1). This modified Sm16(23-117)AA protein was indeed also used in our previous study and was essential for in vitro studies of purified Sm16 in physiological buffers, which revealed efficient binding to cell surfaces and protein-free lipid bilayers (9). Here we show that the modified Sm16 protein forms a 9 ± 1-subunit oligomer with hydrodynamic properties similar to those of Sm16 in crude cercarial extract, thus indicating that the oligomeric state is shared by native Sm16 (Fig. 2).

The Sm16(23-117)AA protein was also functionally compared with the unmodified Sm16 protein by artificially expressing the proteins in the cytosol of transfected human cells and scoring for proapoptotic activity. While this activity is probably of no physiological significance, it still represents a distinctive and potent action of the Sm16 protein. In this experimental setting, which implies modest expression levels, we found that the Sm16(23-117)AA protein was expressed as strongly as the unmodified protein and exerted the same level of apoptosis-promoting activity (data not shown; 9). Thus, given evidently the same oligomeric structure and an unaltered proapoptotic activity, it seems reasonable to assume that replacement of Ile-92 and Leu-93 with Ala in applicable derivatives of Sm16 results in a complete failure to assemble the native approximately nine-subunit oligomer while more centrally located regions are sufficient for trimer formation. The differences in heparin binding between these oligomers (Fig. 3D and E) indicate that the polyanion binding surfaces of Sm16 trimers are masked in the approximately nine-subunit oligomer, which would be consistent with a model in which a nine-subunit Sm16 oligomer is built from three trimers. However, both of these oligomeric forms appeared stable after separation by gel filtration, as indicated by both sucrose gradient sedimentation (Fig. 3C) and a second round of gel filtration (data not shown). We therefore have no direct evidence that the observed trimers have the potential to form the approximately nine-subunit oligomer. Thus, while the present analyses of truncated Sm16 establish a potential to form stable trimers, the significance of trimer formation for the oligomeric configuration of native Sm16 remains to be established.

Short consecutive C-terminal truncations result in a gradual decrease in the approximately nine-subunit oligomer, but as revealed by the Sm16(23-70) protein, removal of the C-terminal half of Sm16 results in a complete failure to assemble the approximately nine-subunit oligomer (Fig. 4A). Provided replacement of Ile-92 and Leu-93 with Ala in applicable derivatives, all C-terminal truncation derivatives were efficiently expressed in E. coli, which also includes the Sm16(23-70) protein, and the purified proteins appeared equally stable (data not shown). Thus, assembly into the approximately nine-subunit oligomer is not of importance for the structural integrity of Sm16. By analyzing the cell surface binding of approximately nine-subunit oligomers isolated by gel filtration, we found a gradual decrease in binding that appeared to correlate with a decreased propensity of the truncated Sm16 protein derivatives to form approximately nine-subunit oligomers. However, since isolated approximately nine-subunit oligomers were used for analysis, our results show that the Sm16 C-terminal end is important not only for oligomerization but also for cell surface binding of the assembled approximately nine-subunit oligomer.

To aid the development of vaccines, both the innate and adaptive immune responses during Schistosoma infections have been extensively studied (for a review, see reference 11). However, although the parasite has evidently developed strategies to avoid the host immune response, the mechanisms and molecules involved remain poorly characterized. The original functional study of the Sm16 protein involved separations of secreted cercarial proteins by denaturing SDS-PAGE, excision of an ~16-kDa region, and subsequent elution of proteins (16). This one-step protocol implies a partial purification of denatured Sm16 monomers, and it is not clear whether the reported activities reflect the activity of the pure native protein. For example, inhibition of antigen-specific T-lymphocyte proliferation was observed, in contrast to the present finding that not even high concentrations of Sm16 have any effect on the anti-CD3 response (Fig. 5). Moreover, it was also reported that Sm16 increased the production of the anti-inflammatory cytokine IL-1RA, which provoked the proposal that Sm16 acts through an IL-1RA-dependent mechanism. This is not supported by our study since recombinant Sm16 produced in P. pastoris actually inhibited the production of IL-1RA in LPS-
poly(I:C)-stimulated human blood cells (Fig. 7). In a subsequent study (17), it was reported that intradermal injection of an Sm16-encoding DNA construct into mice resulted in a detectable suppression of LPS-provoked cutaneous inflammation, as well as a general immunosuppressive activity manifested on the level of T-lymphocyte proliferation. While our analysis of the action of a purified Sm16 protein is certainly consistent with inhibition of a cutaneous inflammation, we did not find evidence of a lymphocyte-directed activity of Sm16.

The present study reveals a potent Sm16-mediated inhibition of the cytokine response induced by two structurally distinct TLR ligands, namely, LPS and the double-stranded RNA mimic poly(I:C). The molecular mechanism was approached by analysis of the IRAK1 protein, which is known to be degraded in response to the activation of a signaling complex directly associated with some of the plasma membrane-located TLRs, such as TLR2 and TLR4 (reviewed in reference 1). Based on specific inhibition of IRAK1 protein depletion in cells stimulated with the TLR4 ligand LPS (Fig. 10B), we present evidence that inhibition is exerted proximal to the TLR complex. Consistent with poor inhibition of the cytokine response to the TLR2 ligand PG (Fig. 9), we did not detect Sm16-mediated inhibition of PG-induced IkB-α or IRAK1 degradation (Fig. 10). While we cannot explain the large difference in the potency with which Sm16 inhibits the response to LPS and PG, these differences still serve to ensure the specificity of the potent inhibition of the LPS response.

Sm16 appears to bind equally well to the surfaces of diverse cell types, which would also be expected if binding were primarily mediated by the demonstrated affinity of Sm16 for lipid bilayers (9). This affinity for lipid bilayers seems likely to be functionally relevant, and our previous fluorescence microscopy analysis of cells cultured with Alexa 488-labeled Sm16(23-117) has indeed revealed efficient plasma membrane binding, followed by Sm16 uptake by endocytosis. The truncated Sm16(23-70) protein was also included in the analysis, and in this case we did not detect plasma membrane binding or endocytosis (9), which is consistent with the present finding that Sm16(23-70) does not detectably inhibit the LPS response (Fig. 6).

Analysis of cells cultured with Alexa 488-labeled Sm16(23-117) indicated that all intracellular fluorescence was confined to intracellular vesicles, and we obtained no evidence that Sm16 penetrates lipid bilayers to escape from endocytotic vesicles to the cytosol (9). This apparent absence of membrane-penetrating properties in Sm16 is indeed consistent with our persistent failure to reproduce the apoptosis-promoting effect of cytosolic expression of Sm16 by adding Sm16 to the culture media of various types of human cells (no effect at 100 μg/ml Sm16; data not shown). Thus, we found no evidence that exogenous Sm16 gains access to the cytosol, which argues against a physiological significance of the previously noted apoptosis-promoting activity of Sm16 artificially introduced into the cytosol (9). Membrane binding in the absence of membrane penetration implies that Sm16 will remain on the extracellular side of cellular lipid bilayers.

We observed large differences in the potency of inhibition of the four distinct TLR ligands used in this study (Fig. 7 and 9), which suggests that Sm16 is not a general inhibitor of TLR signaling. However, it is notable that Sm16 is a potent inhibitor of the poly(I:C) response (Fig. 7 and 9), which is mediated by a distinct receptor only found in intracellular vesicles, namely, TLR3 (reviewed in reference 1). Given the efficiency with which membrane-bound Sm16 is taken up by endocytosis and the evidence of accumulation in intracellular vesicles (9), it seems reasonable to assume that Sm16 exerts its inhibition of TLR3 signaling from the endoplasmic luminal side of the vesicle by the same general mechanism by which Sm16 inhibits LPS signaling by plasma membrane-located TLR4. This implies that Sm16 may inhibit TLR function through some type of interference with ligand-induced TLR signaling. Such a mechanism of action appears consistent with our observation that various TLR ligands are inhibited with different degrees of potency.

Inhibition of TLR signaling during skin penetration may represent a strategy for avoidance of inflammatory reactions caused by tissue damage and/or cercaria-derived TLR ligands. There is indeed evidence of both TLR stimulatory ligands (reviewed in reference 14) and activities that suppress TLR function (20) among helminth parasites. However, to our knowledge, the present studies of the Sm16 protein provide the first example of TLR inhibition by a purified parasite-derived protein. The Sm16 protein is abundant (3 to 4% of acetylub gland secretions) (5), and the demonstrated binding to cell membranes (9) will prevent diffusion. Thus, there are reasons to assume local concentrations of Sm16 sufficiently high to suppress activation of the innate immune response in the dermis proximal to the site of cercarial entry.

ACKNOWLEDGMENTS

We are indebted to Cecilia Thors, Swedish Institute for Infectious Disease Control, Stockholm, for providing cercariae. This work was supported by the Swedish Research Council.

REFERENCES