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The Salivary Scavenger and Agglutinin in Early Life: Diverse Roles in Amniotic Fluid and in the Infant Intestine

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The salivary scavenger and agglutinin (SALSA), also known as gp340 and dmbt1, is an antimicrobial and inflammation-regulating molecule located at the mucosal surfaces. The present study revealed that SALSA was present in the amniotic fluid (AF) and exceptionally enriched in both meconium and feces of infants. Based on immunological and mass spectrometric analysis, SALSA was estimated to constitute up to 4–10% of the total protein amount in meconium, making it one of the most abundant proteins. SALSA proteins in the AF and intestinal samples were polymorphic and exhibited varying polypeptide compositions. In particular, a different abundance of peptides corresponding to functionally important structures was found in the AF and intestinal SALSA. The AF form of SALSA had a more intact structure and contained peptides from the zona pellucida domain, which is involved in cell differentiation and oligomerization. In contrast, the intestinal SALSA was more enriched with the scavenger receptor cysteine-rich domains. The AF, but not the meconium SALSA, bound to Streptococcus pyogenes, S. agalactiae, S. gordoni, and Escherichia coli. Furthermore, differential binding was observed also to known endogenous ligands C1q, mannose-binding lectin, and secretory IgA. Our results have thus identified mucosal body compartments, where SALSA is particularly abundant, and suggest that SALSA exhibits varying functions in the different mucosal locations. The high levels of SALSA in AF and the infant intestine suggest a robust and important function for SALSA during the fetal development and in the mucosal innate immune defense of infants. The Journal of Immunology, 2014, 193: 000–000.

Innate immunity is extremely important for the mother in the course of maintaining a healthy pregnancy, as well as for the infant in the early stages of life. The mucosal surfaces are sites with a very tight yet dynamic regulation of the immune defense system (1, 2). Newborns have not yet developed a fully functional adaptive immune system at the time of birth. Therefore, they rely greatly on the innate immune system (3). In early life, both the fetus and newborn experience alternating immune challenges (4). These include avoidance of a harmful immune response from the mother, which could lead to preterm birth, protection against infection, and coping with the transition from a mostly sterile intrauterine environment into a world that is full of foreign Ag challenges. The latter becomes particularly important during the initial colonization of the skin and intestinal tract through the first months of life (5, 6).

The salivary scavenger and agglutinin (SALSA), also known as gp340, salivary agglutinin, and deleted in malignant brain tumor 1 (dmbt1), is a 340-kDa glycoprotein (GenBank accession number BAA78577.1) (7, 8). SALSA is expressed by a variety of mucosal tissues throughout the body. It is associated with the epithelial layer in the lungs, mouth, trachea, gastrointestinal tract, and vagina (9–13). Several soluble forms of SALSA have been found in body fluids lining the mucosal surfaces such as saliva, lacrimal fluid, and pancreatic juice (7, 14, 15). Notably, SALSA has not been observed in human blood or plasma.

Multiple functions have been suggested for SALSA including roles in epithelial differentiation and innate immunity at the mucosal surfaces (10). The immune functions of SALSA are apparent through its well-established ability to bind and agglutinate a broad spectrum of both Gram-negative and Gram-positive bacteria, as well as viruses (16–22). In several cases, such as with Salmonella enterica, Streptococcus mutans, HIV-1, and influenza A virus, SALSA has been shown to have a direct effect on controlling the infection (16, 18, 23, 24). SALSA’s antimicrobial effects are mediated in concert with other innate immune molecules such as mucin 5B, IgA, and surfactant proteins A and D, all of which act as endogenous ligands for SALSA (7, 25–27). The complement components C1q and mannose-binding lectin (MBL) are other targets for SALSA. Through these interactions, SALSA has been shown to regulate complement activation on surfaces and in solution (28).

Alongside the role of SALSA in innate immunity, a function in epithelial and stem cell differentiation has been suggested. Much
of this evidence comes from work with animal orthologs of SALSA, such as rabbit hensin and mouse CRP-ductin (29, 30). Fewer studies have been performed in humans. However, a role in the epithelial development was suggested based on SALSA’s localization and increased expression in both fetal skin, lung, and gut compared with adults (8, 10).

SALSA belongs to the scavenger receptor cysteine-rich (SRCR) protein family. Its N terminus contains up to 13 SRCR domains repeat separated by scavenger interspersed domains. These are followed by two C1r/C1s Uegf Bmp-1 domains surrounding the 14th SRCR domain and finally a zona pellucida (ZP) domain (9). The dmb1 gene undergoes alternative splicing giving rise to differentially sized mRNAs encoding 8–13 N-terminal SRCR domains (10, 31). At the protein level, two different isoforms have been identified in both saliva and lacrimal fluid (7, 14). One study has estimated that up to 25% of the molecular mass of SALSA is made up of carbohydrate. The observed protein polymorphism is thus a consequence of variation in both the protein core and the glycosylation patterns (32, 33).

Innate immunity is extremely important for both the mother, in maintaining a healthy pregnancy and subsequent lactation, as well as for the infant in the early stages of life. In the current study, exceptionally high levels of SALSA were found immediately before birth (in amniotic fluid [AF]) and shortly after birth (in meconium and feces after 1 wk). The SALSA proteins in the AF and intestinal samples were found in multiple forms and with varying functional activities. Different binding abilities to both microbial and endogenous ligands were found between SALSA in the fairly sterile microenvironment surrounding the fetus in the womb and SALSA in the intestine, when the infant encounters a colonizing microbiota for the first time. Our results thus indicate SALSA as an important innate defense molecule in early life.

Materials and Methods

Samples

AF, meconium, and fecal samples (n = 9 for each) were collected at the Obstetrics or Neonatology Units of Hospital Universitario Doce de Octubre (Madrid, Spain). AF was collected before rupture of membranes either during caesarean section (n = 4) or intravaginally (n = 5) before delivery. Meconium was collected from term newborns after spontaneous evacuation within the first 2 h from birth and before feeding was started. To avoid potential bacterial contamination arising from the contact between meconium and perianal skin/nappies, the outer surface of each meconium sample was removed using a laser scalpel. Then, an internal meconium portion was submitted to analysis. The fecal samples were collected directly into sterile containers 1 wk after birth. All sample types were stored at −80°C after collection. All infants were delivered by healthy mothers after a normal pregnancy and breastfed after birth. All parents provided written informed consent, and the study was approved (protocol B-06/262) by the Ethical Committee in Human Clinical Research of Hospital Clinico San Carlos (Madrid, Spain).

Protein extraction

Proteins were mechanically extracted from dried AF or unprocessed meconium and fecal material by bead beating as described previously (34). AF aliquots (50 μl) were dried by speedvac and resuspended into PBS (pH 7.4) followed by sonication. Thawed meconium or fecal material (125 mg) was resuspended in 375 μl PBS and sonicated. Then, all samples were subjected to a FastPrep 24 (MP Biomedicals) according to the manufacturer’s instructions. The resulting protein extracts were stored at −80°C.

Western blotting

To visualize SALSA samples were diluted diversely in TBS (140 mM NaCl and 5 mM Tris [pH 7.4]) containing 1 mM Ca2+ (TBS/Ca) (AF7 and AF8, 1:10; M7, AF9, and M9, 1:20; F7, M8, and F9, 1:50; and F8, 1:100). After dilution, all samples were mixed with nonreducing SDS-PAGE loading buffer and loaded (10 μl) onto a 4–12% gradient SDS-PAGE gel (Life Technologies). The proteins were blotted onto a nitrocellulose membrane (Life Technologies). Nonreceptor to the membrane was blocked with 5% nonfat dry milk in PBS with 0.05% Tween 20 (PBS/Tween). Mouse monoclonal anti-SALSA Ab (Hyb 213-06; Bioprotein, Gentofte, Denmark) was added to the milk solution (1:10,000) and incubated overnight at 4°C. After washing, a secondary HRP-conjugated rabbit anti-mouse IgG Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) was added (1:10,000 in PBS). The bands were visualized by electrochemiluminescence.

Glycoprotein and lectin staining

SALSA was purified from AF as described (17). Purified SALSA (10 μl) was loaded in triplicate onto 7.5% SDS-PAGE gel. After electrophoresis, the gel was cut in three parts. One part was stained for protein with silver nitrate and the second part for glycoproteins with periodic acid–Schiff reagent (Glycoprotein Staining Kit; Pierce) according to the manufacturer’s instructions. The last part of the gel was blotted on polyvinylidene difluoride membrane (Amersham) for staining with DIG-labeled sialic acid–specific Sambus Nigra lectin (DIG Glycan Differentiation Kit; Boehringer Mannheim). Nonspecific binding was blocked with 3% BSA and the membrane incubated with the lectin (1:1000) overnight at 4°C. Lectin binding was detected with anti–dig-AP according to the manufacturer’s instructions. Bovine submaxillary gland mucin and unrelated bacterial surface proteins were used as positive and negative controls, respectively, in the glycoprotein and lectin staining assays.

Quantification of SALSA in protein extracts by ELISA

To quantify the concentration of SALSA in the different extracts, the extracts were diluted in TBS/Ca. The diluted samples were coated directly onto Maxisorp plates (Nunc, Roskilde, Denmark). SALSA purified from saliva was used as a protein concentration standard. After coating, the plates were blocked with 5% nonfat milk in TBS/Ca. The plates were washed with TBS/Ca containing 0.05% Tween-20 (TBS/Ca/Tween). SALSA levels were detected using monoclonal anti-SALSA (0.05 μg/ml) and HRP-conjugated rabbit anti-mouse Abs (1:10,000 in TBS/Ca). OPD tablets (DakoCyto- mation, Glostrup, Denmark) were used for development, and the color reaction was measured with an iEMS Reader MF (Labsystems, Espoo, Finland) at an OD of 492 nm.

Liquid chromatography–tandem mass spectrometry

Protein extracts were separated on a one-dimensional polyacrylamide gel. By using a prestained marker (Bio-Rad) as an m.w. indicator, each sample lane on the stained gels was further divided into four regions. For fecal and meconium samples, these regions roughly ranged from: >250–75 kDa, 75–50 kDa, 50–30 kDa, and 30 to <10 kDa. For AF samples, the gel lanes were cut from >250–70, 70–60, 60–35, and 35 to <10 kDa. NanoLC and LTQ-Orbitrap-MS analyses, including quality checks and machine calibrations, were performed as described (35). For tandem mass spectrometry (MS/MS) spectral identifications, an in-house database was constructed containing a comprehensive set of protein sequences that can be expected to occur in the (infant) gastrointestinal tract.

Bacterial binding of SALSA

Group A Streptococcus (GAS; ATCC 19615), group B Streptococcus (GBS), a clinical blood isolate, isolated and identified at the Helsinki University Central Hospital Laboratory, and S. gordonii, D1L Challos (22), were grown in Todd-Hewitt media overnight at 37°C. Escherichia coli (urine isolate) and Salmonella serovar Typhimurium (fecal isolate) were grown overnight at 37°C with shaking in Luria broth. AF, meconium, or fecal protein extracts were diluted in TBS/Ca to a final concentration of 0.5 μg/ml SALSA and incubated with 107 bacterial cells in a 50-μl suspension for 1 h at 37°C. After incubation, the bacteria were centrifuged at 10,000 × g. The supernatants were collected, and the bacteria were washed three times in TBS/Ca. Bound SALSA was eluted by incubating the bacteria in 50 μl nonreducing SDS-PAGE loading buffer (Life Technologies) containing 10 mM EDTA. Binding was visualized by Western blotting. For each sample, SALSA in the starting material was compared with SALSA in the supernatant after absorption with bacteria and SALSA eluted from the surface of the bacteria. To control that no factors in meconium were inhibiting the SALSA binding, AF was mixed with varying amounts of meconium. We thus analyzed whether the eluted amount of SALSA protein was affected by an increasing amount of meconium.

Endogenous ligand binding of SALSA

Binding of SALSA from AF and meconium was tested to known ligands of salivary SALSA: secretory IgA (Sigma-Aldrich), C1q (Quidel), and...
recombinant MBL (28). Each protein (1 μg/ml) was coated onto Maxisorp plates (Nunc, Roskilde, Denmark). After coating, the plates were blocked with 1% BSA in TBS/Ca/Tween and then washed with TBS/Ca/Tween. AF and meconium samples were diluted into a SALSA concentration of 1 μg/ml and incubated in the wells 1 h at 37°C. SALSA binding was detected using mouse monoclonal anti-SALSA (0.05 μg/ml) and HRP-conjugated rabbit anti-mouse Abs (1:10,000 in TBS/Ca). Binding was measured as described above for quantification of SALSA in protein extracts.

Results

SALSA in AF, meconium, and infant feces

To improve our understanding of the role of SALSA in early life, we studied its presence in AF, meconium, and feces (1 wk after birth) samples serially collected from a cohort of nine infants. This allowed a comparison of biological material from mucosal surfaces of an individual both within the uterus and in the early days of life. Proteins were extracted from the AF, meconium, and feces samples. To characterize the SALSA protein in the samples, they were analyzed by Western blotting for individual differences in size and band patterns. Examples from three newborn individuals are displayed and compared with SALSA in saliva from a healthy adult in Fig. 1.

Bands immunoreactive with SALSA were found in all samples in the area of the expected 340 kDa under nonreducing conditions. However, there was a great variation in the appearance of SALSA, not only from individual to individual in the same body compartment, but also from different compartments within the same individual. This indicated heterogeneity in size and composition of the proteins. In AF, SALSA appeared either as one thick band or as a smeared double band (case 7). Cases 8 and 9 seemed to have only the higher m.w. thick band. The meconium SALSA bands presented as much wider smears on the blot compared with SALSA in both AF and fecal samples. Within the smears, some individual bands were observed. For example, in case 7, meconium SALSA appeared to have a double band with the higher one at ~200 kDa and the lower one at ~150 kDa. For both case 7 and 9, the meconium SALSA smear clearly appeared at a lower m.w. area than AF SALSA. In case 8, SALSA in meconium appeared as a higher m.w. band than in AF. Yet, there was a wide smear extending from the high m.w. area to ~150 kDa. Finally, the bands observed in the fecal samples appeared as more distinct bands, similar to those in the AF samples.

A positive reaction was observed, when AF samples were stained with periodic acid-Schiff and the sialic acid–specific Sambucus nigra lectin. This indicated that SALSA is glycosylated and contains carbohydrates with terminal sialic acids (data not shown). The heterogeneity in size and appearance of the SALSA bands suggested both structural differences among the proteins detected in the different compartments as well as variations in glycosylation or other posttranslational modifications.

To determine the levels of SALSA in the different compartments during early life, the protein extracts were analyzed by ELISA or digested with trypsin and subjected to liquid chromatography-mass spectrometry (LC-MS/MS). As expected, we were able to identify and semiquantify peptides that matched to the SALSA protein in all 27 samples and thus in all 3 sample types. The LC-MS/MS analysis was used to compare the relative abundances of SALSA based on the number of spectra matching to SALSA-specific peptides in relation to the total amount of protein spectra in a given sample (Fig. 2A). The relative abundance of SALSA was 0.53% for AF, 4.16% for meconium, and 2.81% for feces. Thus, the levels of SALSA were significantly higher in both meconium and feces compared with AF (p = 0.002). The ELISA analysis provided actual protein concentration measurements in the protein extracts (Fig. 2B). The average values were 2.1 μg/ml (range 0–11.5 μg/ml) for AF, 45.8 μg/ml (range 2.8–294.6 μg/ml) for meconium, and 22.4 μg/ml (range 0.1–62.9 μg/ml) for feces. The amounts of both meconium and feces produced by the newborns vary greatly. The total amount of amniotic fluid is, however, much larger. With an average total volume of 700 ml at birth, the amniotic fluid contains ~1.5 mg of SALSA (36). Case 1 showed very high levels of SALSA in meconium. However, in this case there were no abnormalities during the pregnancy, delivery, or the first week of life.

Analysis of peptide compositions

Based on the proteomics data, we were able to further analyze the polypeptide compositions of SALSA molecules from different sources. Fig. 3 shows a heat map of the 28 identified peptides matching to SALSA. For each sample, the relative quantities of the peptides found within the sample are displayed. Based on this, the samples were clustered. Very interestingly, the AF samples clustered together (the left side of the heat map), whereas meconium samples clustered into another distinct group (right side). Fecal samples were spread out across the horizontal axis. However, except for F1, F4, and F6, most of the fecal samples clustered separately from the AF samples.

Peptides 7–9, 10, and 25 (marked with arrows in Fig. 3; displayed in full in Table I) were most significantly different in their relative abundance (p < 0.00002) in AF compared with the intestinal samples. Thus, they were analyzed in more detail. Peptides 10 and 25 were relatively more abundant in the AF samples, whereas peptides 7–9 were relatively less abundant in the AF samples. The specific clustering indicates a difference in the SALSA proteins from AF versus those in meconium and feces in the particular areas of the protein containing these peptide sequences. Alternatively, the proteins could become differentially processed in the different environments, for example, by proteolytic or glycolytic enzymes and/or binding partners.

Structural differences of AF versus intestinal SALSA

Comparative structural analyses were carried out to get more insight into the locations of the discriminatory peptides of AF and intestinal SALSA. In Fig. 4A, the domain organization of SALSA is displayed (9). The specific locations of the five individual peptides are displayed above the respective domains, where they are found in the SALSA protein. Peptides 7–9 have the same core sequence but vary in 1 to 2 aa. There is one representative of peptide 7, 8, or 9 in every SRCR domain except in domain 14.
Measurements were performed in duplicates and repeated three times. Relative levels of SALSA were seen in AF samples compared with meconium and feces. (i.e., bacterial binding via the SRCR domain and extracellular in regions with a direct link to suggested protein functions between AF- and intestine-derived (meconium and fecal) SALSA sequences thus suggests the existence of structural differences for SALSA. Our analysis of the abundance of certain peptide the high level of similarity, we suspect that the same is the case providing the specificity of the egg coat assembly (38). Due to in the ZP domain–mediated protein dimerization function, thus of the cZP3 structure suggested that this area is directly involved in the ZP domain. The three-dimensional structures of some SRCR and ZP domains have been determined, allowing comparison between the peptide sequences and the structures. In Fig. 4B, the crystal structure of the group A SRCR of the Mac2-binding protein is displayed (37). Peptide 10, present in SRCR domains 1–13 and found to be more abundant in the AF SALSA, is located in an extended loop between aa 56 and 69 in the SRCR model domain of the Mac2-binding protein. No known function has been ascribed to this particular loop. However, when the remaining peptides (7–9, 25) were compared with the homologous SRCR and ZP structures, they could be potentially linked to established SALSA functions. SALSA is known to bind to a broad range of bacteria, and a very specific peptide sequence responsible for these interactions, RVEVLYxxxSW, has been identified within the SRCR domain (21). This sequence is highlighted in yellow (68% homology to SALSA) in Fig. 4B. Interestingly, peptides 7–9 (highlighted in red), which were relatively less abundant in AF, overlapped with the bacterial binding sequence. The N-terminal QSW of these peptides matched the xSW from the bacterial binding sequence. The overlap is shown in orange in Fig. 4B.

In Fig. 4C, the crystal structure of a full-length ZP domain from chicken sperm receptor 3 (cZP3) is displayed (38). This homologous structure was used as a model for the SALSA ZP domain. The two proteins are similar (56% identity) in the region of peptide 25. The sequence of the AF-abundant SALSA peptide matched a loop extending from the ZP-c subdomain. An analysis of the cZP3 structure suggested that this area is directly involved in the ZP domain–mediated protein dimerization function, thus providing the specificity of the egg coat assembly (38). Due to the high level of similarity, we suspect that the same is the case for SALSA. Our analysis of the abundance of certain peptide sequences thus suggests the existence of structural differences between AF- and intestine-derived (meconium and fecal) SALSA in regions with a direct link to suggested protein functions (i.e., bacterial binding via the SRCR domain and extracellular communication/polymerization via the ZP domain).

Thus, the differences in these peptides simply represent variations in the SRCR domains. Peptide 10 was found within all SRCR domains, again with the exception of domain 14, whereas peptide 25 was only found once in the entire protein, within the ZP domain.

The Functional differences between AF and intestinal SALSA To directly address the question whether AF and intestinal SALSA have functional differences, we tested their binding to three different types of streptococci, GAS, GBS, S. gordonii, E. coli (an intestinal commensal), and S. Typhimurium (an intestinal pathogen). The bacteria were incubated with protein extracts diluted into a SALSA concentration of 0.5 μg/ml. After centrifugation, the bound SALSA was eluted using SDS-PAGE loading buffer containing 10 mM EDTA. The ability of the bacteria to absorb SALSA from the various solutions is visualized in Fig. 5 by Western blotting. The binding patterns are representatives of two to six tested individuals.

SALSA from AF bound to GAS, GBS, S. gordonii, and E. coli. The SALSA band disappeared after incubation with the bacteria in all cases and was found after elution from the bacterial surfaces. In contrast, SALSA from meconium did not bind to any of the bacterial strains but remained in the solution. Interestingly, S. Typhimurium did not bind any type of SALSA. The fecal samples showed greater variation in the bacterial binding abilities. From some samples, SALSA did not bind to any of the tested bacteria. However, from other samples, SALSA bound to GAS and S. gordonii. Some binding was also observed to GBS. However, binding was not strong enough to deplete SALSA from the fecal suspension used in the assay. To control that no factors in meconium were inhibiting the SALSA binding, AF was mixed with varying amounts of meconium. Regardless of the concentration of meconium, the same amount of SALSA became bound and was eluted from the bacteria, showing no effect of meconium products on the binding of AF-SALSA to the bacteria (data not shown).

Given the clear variation in binding abilities of SALSA from AF and meconium to bacteria, we further tested SALSA interactions with known endogenous protein ligands of salivary SALSA. We compared SALSA binding from several AF and meconium samples to secretory IgA, MBL, and Clq (Fig. 6). SALSA from all AF samples bound clearly to IgA, MBL, and Clq. No binding to any of the ligands was observed by SALSA from any of the meconium samples.

Discussion
After performing a screen of various body fluids and biological materials from mucosal surfaces, we were able to identify the SALSA protein in AF and meconium. To our knowledge, this is the
The sequences of the respective peptides are shown in Table I. Observed for five peptides: 7, 8, 9, 10, and 25 (marked with red arrows). Abundance between the AF group and the intestinal samples is particularly clustered (top horizontal axis). All AF samples cluster to the left side of the heat map together with fecal samples F1, F4, and F6. A differential abundance in AF and meconium/feces (p < 0.00002). X indicates leucine or isoleucine.

A key finding of the current study was the abundance of SALSA in the meconium and feces in early infancy, averaging ~3 to 4% (range 1–10%) of the total protein in these samples. Such abundance underlines the importance of this protein at this early stage of life. Both the relative abundance studies and the actual concentration measurements of SALSA revealed that the levels of SALSA in the extracts from meconium and fecal samples were notably higher than those in the AF samples. On the average, the concentrations of SALSA were 2.1 µg/ml for AF, 45.8 µg/ml for meconium, and 22.4 µg/ml for feces. However, AF constitutes one of the largest physiological reservoirs of SALSA (~1.5 mg) because of its large volume during late pregnancy (~700 ml at birth) (36). After birth, the largest pool of SALSA apparently resides in the gastrointestinal tract (9, 10).

Initially, Western blotting was used to analyze potential variations in the size of the SALSA protein among individuals and among the different types of samples collected. A band was observed in the area of 340 kDa for all AF, meconium, and fecal samples (Fig. 1). This correlated well to observations made in other body fluids such as saliva and lacrimal fluid (Fig. 1) (7, 14). Unfortunately, direct comparison with SALSA in saliva from the individuals in this cohort was not possible, because salivary samples were not available for the study. Individual differences were observed when the same types of biological samples from different individuals were compared, especially in meconium and feces. Size polymorphisms of SALSA have been described before and used to classify individuals into four groups (groups I–IV) (20, 33). This grouping was shown to correlate with Lewis-Ag expression, secretor status, and bacterial binding properties (33). Individual variations in SALSA observed in AF, meconium, or feces may reflect such grouping.

Differences in the SALSA band patterns across different tissues within the same individual were also observed in this study. Previously, two isoforms of SALSA have been described in tear fluid from the same individual, but SALSA has never before been compared in different mucosal compartments from the same person (14). In this study, a clear difference among the proteins found in AF, meconium, and feces were observed. Overall, SALSA in meconium appeared with a lower m.w. than that in AF. The same seemed to be the case for fecal SALSA. However, the difference to AF SALSA was not so clear cut. The origin of SALSA, both in the fetal gut and within the amniotic cavity, remains unclear. AF is a bioactive medium with constituents actively secreted by cells lining the amniotic cavity and, during early stages, liquid filtered from the maternal blood. As gestation progresses, AF includes a significant volume from fetal urine (up to 50%). However, SALSA could not be detected in adult urine samples (data not shown). SALSA in AF may be secreted into AF by either the amniotic epithelial cells or the prenatal fetus. Meconium is to a great extent a concentrate of the swallowed AF during the fetal development, and AF could thus be the source of SALSA in meconium. In the infant intestine, as well as during later stages, production by the intestinal epithelium is probably the most important source of SALSA.

In addition to Western blotting, an analysis using proteomics techniques revealed peptide variations in functionally relevant regions of the SALSA protein. A mass spectrometric analysis of the relative abundances of the 28 SALSA-related peptides revealed sample-specific patterns. Peptides of AF samples clustered clearly separately from the meconium and fecal ones. Peptides 7–10 and

The first description of the presence of SALSA in these biological materials. Previous studies have attributed a role for SALSA in both innate immunity and epithelial differentiation at mucosal surfaces (10). Given the high degree of cell differentiation in general during fetal maturation and the importance of innate immunity at both the fetal and neonatal stages, SALSA may play key roles in these processes during early life. During prenatal life, AF is constantly surrounding the fetal skin and mucosal surfaces of the nasal and gastrointestinal tract. To get a broad understanding of SALSA in early life, we included samples from mucosal surfaces at both the fetal stage (AF) and the infant stages (meconium and fecal samples).

### Table I. Peptide sequences matched to SALSA

<table>
<thead>
<tr>
<th>Peptide No.</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>GSWGTVCDSDWDTDANVVCRR</td>
</tr>
<tr>
<td>8</td>
<td>GSWGTVCDSDWTDNDANVVCRR</td>
</tr>
<tr>
<td>9</td>
<td>GSWGTVCDSDWTDNDANVVCRR</td>
</tr>
<tr>
<td>10</td>
<td>FGQGSPXVXDDVR</td>
</tr>
<tr>
<td>25</td>
<td>SGCVRDRTYGPYSPSXR</td>
</tr>
</tbody>
</table>

Specific amino acid sequences of peptides matching to SALSA in the LC-MS/MS analysis. Displayed are only the peptides with a very significantly different relative abundance in AF and meconium/feces (p < 0.00002). X indicates leucine or isoleucine.
25 together cover three distinct regions within the SALSA protein (Fig. 4). Peptides 7–9 were relatively less abundant in the AF SALSA compared with intestinal SALSA, whereas peptides 10 and 25 were relatively more abundant. It has long been known that SALSA binds to and agglutinates several types of bacteria in the mouth and in the gut (16–21). The broad bacterial binding property has been assigned to one particular peptide sequence found in the SRCR domains (RVEVLYxxxSW) (21). Three amino acids (xSW) in this sequence overlap with the region that was found to be relatively more abundant in the intestinal SALSA than in AF SALSA. Such a structural difference could indicate an altered bacterial binding ability of the intestinal SALSA and/or a difference in the structural integrity of the intestinal and AF forms of SALSA. A difference became even more apparent when the peptide enriched in the AF samples (peptide 25) was considered. The sequence of this peptide was found in the ZP domain only. ZP domains are found in hundreds of extracellular matrix proteins. Often these proteins polymerize through the ZP domain into fibrils or matrices and aid in transforming the cell shape and creating polarization (39). This has been shown for hensin, the SALSA-homolog in rabbits (40). Dmbl1-knockout mouse embryos were found unable to induce columnar epithelia, which is a critical step during embryonic development. As a consequence, the mice died at an early time point (41, 42). Polymerization of SALSA through the ZP domain could as well affect the avidity of interactions with bacteria in the gut. Both soluble and extracellular matrix-bound SALSA would be monomeric in the absence or by the cleavage of the ZP domain. This could weaken and even abolish the bacterial binding ability. The apparent presence or absence of the ZP domain in AF SALSA and intestinal SALSA, respectively, could therefore easily have functional relevance.

Despite the different relative abundances of peptides of the various forms of SALSA, differences at the primary amino acid sequence level are unlikely. Peptides 7–10 are all located within the highly conserved SRCR domains. Each SRCR domain is expressed from a single exon, and therefore, variations within the domains are not expected. However, at the transcriptional level, several isoforms have been described, all showing varying num-

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**FIGURE 4.** Structural overview of the SALSA protein. The peptides highlighted in Fig. 3 and Table I were located in the structure of the SALSA protein. (A) SALSA is comprised of 13 N-terminal SRCR domains, 2 C1r/C1s Uegf Bmp-1 domains surrounding the 14th SRCR domain, and a C-terminal ZP domain (9). The peptides with varying abundance are displayed above the domains of SALSA where they were found. The relative abundances of the peptides found in AF or intestinal (meconium and fecal) samples are indicated. The peptides relatively less abundant in AF samples (peptides 7–9) all represent a particular sequence of the SRCR domain. This sequence is present in all SRCR domains except 14. Peptides 10 and 25 were most abundant in the AF SALSA protein. Peptide 10 was found in each SRCR domain except domain 14, and peptide 25 was found only in the ZP domain. (B) Location of peptides 7–9 (red) in a crystal structure representation of the SRCR domain of Mac-2 binding protein (37). These peptides are partially overlapping with the SRCR peptide sequence responsible for the bacterial binding of SALSA (yellow). The three peptides 7–9 are identical in their N-terminal region. This region overlaps with the GSW of the bacterial binding sequence (orange). (C) Location of peptide 25 (purple) in a crystal structure model of cZP3 (38). This sequence is part of the ZP-c subdomain, a structure believed to be directly involved in the protein dimerization function mediated by the ZP domain.
differences in the ability to bind bacteria. (F) SALSA from feces showed individual differences in the ability to bind bacteria. SALSA from feces showed individual differences in the ability to bind bacteria. SALSA from meconium does not bind to any of the tested bacteria. SALSA from meconium does not bind to any of the tested bacteria. SALSA from meconium did not bind to any of the tested proteins. These data show that the structural differences can affect the function of the SALSA protein. Apparently, SALSA in AF is intact and fully functional. However, SALSA recovered from meconium and some fecal samples is altered and does not have the bacterial and endogenous protein binding property.

The contribution of amniotic cavity and fetal/infant gut to the total SALSA concentration in the analyzed samples may vary on an individual basis. As a consequence, site-specific polymorphisms, cleavage patterns, oligomerization, and/or glycosylation and other posttranslational modifications may explain the SALSA protein variations among the different types of samples analyzed in this study. Glycosylation and other posttranslational modifications may interfere with trypsin digestion. These have been described in SALSA from bronchoalveolar lavage, saliva, and lacrimal fluid (44, 45). The size variations may therefore be related to differential posttranslational modifications within the different protein areas making them more or less susceptible to cleavage by trypsin. Trypsin is naturally present in the intestine and could thus influence the bacterial binding properties of SALSA in vivo (46). The ZP domain could be proteolytically cleaved in the intestinal environment and thus be lost during the protein extraction.

A prominent role for SALSA at the mucosal surfaces has been suggested, including an involvement in inflammatory conditions such as Crohn disease and ulcerative colitis (47). For infants, the importance has also been shown through its altered regulation in preterm infants with neonatal infections (48). In the current study, we describe for the first time, to our knowledge, the presence of SALSA in both AF and meconium. The very high protein levels observed support a crucial role for SALSA in the early life. Innate immune clearance functions and cell-differentiation processes are vital for fetal development, and SALSA may be involved in both. SALSA in AF could be involved in epithelial cell differentiation and extracellular matrix binding through the ZP domain. At the same time, it could play an important role in keeping the amniotic cavity sterile by agglutinating bacteria and activating the complement system. The observed loss of bacterial and endogenous
ligand binding in the intestine may indeed be a result of regulation of the protein function at the mucosal surface (e.g., by proteolytic or glycolytic cleavage). In the early neonatal life, the mucosal surfaces throughout the body undergo massive transformation. The initial colonization of the body starts, and a commensal healthy microbiome is acquired in concert with the development of the body’s own immune system. The differences in bacterial binding of SALSA, observed in the fecal samples, may be part of the regulation of the colonizing microbiota. The very early presence of SALSA and its bacterial binding functions suggest that it may be a key player in the selection of a healthy intestinal commensal microbiome.

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Disclosures

The authors have no financial conflicts of interest.

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