Requirement of the Epithelium-specific Ets Transcription Factor Spdef for Mucous Gland Cell Function in the Gastric Antrum

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Mucus-secreting cells of the stomach epithelium provide a protective barrier against damage that might result from bacterial colonization or other stimuli. Impaired barrier function contributes to chronic inflammation and cancer. Knock-out mice for the epithelium-specific transcription factor Spdef (also called Pde) have defects in terminal differentiation of intestinal and bronchial secretory cells. We sought to determine the physiologic function of Spdef in the stomach, another site of significant levels of Spdef expression. We used in situ hybridization and immunohistochemistry to localize Spdef-expressing cells in the mouse stomach; targeted gene disruption to generate mice lacking Spdef; and histologic, immunologic, and transcriptional profiling approaches to determine the requirements of Spdef in stomach epithelial homeostasis. In wild-type mice, Spdef RNA and protein are expressed predominantly in mucous gland cells of the antrum and in mucous neck cells of the glandular corpus. Within 1.5 years, nearly half of homozygous mutant mice developed profound mucosal hyperplasia of the gastric antrum. Submucosal infiltration of inflammatory cells preceded antral hyperplasia by several weeks. The absence of Spdef impaired terminal maturation of antral mucous gland cells, as reflected in reduced expression of Muc6 and Tff2 and reduced numbers of secretory granules. Antral gene expression abnormalities overlapped significantly with those in Spdef−/− colon, including genes implicated in secretory granule traffic and functions. Spdef is required for terminal maturation of antral mucous gland cells to protect animals from gastric inflammation and resulting hyperplasia. These requirements parallel Spdef functions in secretory intestinal cells and suggest a common molecular mechanism for maturation of gastrointestinal secretory lineages.

The Ets protein family in humans and mice contains nearly 30 transcription factors. Loss of several family members in knock-out mice reveals their critical requirement in embryogenesis, morphogenesis, and cell differentiation (1–3). Deregulated expression or activation of Ets factors is also linked to various human cancers (4, 5), highlighting their importance in disease. Ets proteins were first studied in detail in lymphocytes and neurons (6, 7). Their role in epithelial cells gained attention after we and others identified four additional Ets factors: Elf3 (Ese1), Elf5 (Ese2), Ehf (Ese3), and Spdef (Pde) (8–10). These factors are virtually restricted to epithelial cells, but each shows a distinct tissue distribution, with further restriction to distinct cell subsets or differentiation stages (11), suggesting specialized roles in different lineages.

Spdef is unique among Ets proteins for its distinct DNA binding specificity and restricted expression in hormone-regulated prostate, mammary, endometrial and ovarian epithelia, salivary gland, trachea, lungs, and digestive tract (9, 12). Spdef function has been studied in cancer cell migration and prostate, ovarian, and breast cancer (13–16); its role in normal tissues is starting to emerge. Exposure to intratracheal allergens or IL-13 leads to excess mucus production as a result of Spdef-dependent goblet cell differentiation, whereas Spdef expression in transgenic bronchial epithelium causes goblet cell hyperplasia and mucus hypersecretion (17), defects related to human lung diseases. Spdef−/− mice show defective differentiation of pulmonary goblet cells and intestinal Paneth and goblet cells, together with deregulation of secretory cell-specific genes (18, 19).

After gastrointestinal mucosal progenitors commit to a particular cell fate, they undergo additional differentiation steps before acquiring the unique ability to absorb nutrients or secrete enzymes, mucus, acid, hormones, or antimicrobial peptides. Combinations of cell type-specific and broadly expressed transcription factors regulate each step, as highlighted in recent reports of the roles of transcription factors Foxq1 and Mist1 in specific aspects of gastric foveolar (pit) and corpus mucous neck cells, respectively (20, 21). In the intestine, Spdef−/− mice...
show reduced mucus production and low granule numbers in goblet and Paneth cells, respectively, implicating Spdef in terminal differentiation of these secretory cells (19, 22). We generated an independent null allele for Spdef, and here we report on the requirement for this epithelial Ets factor in the mammalian stomach. Antral mucosal hyperplasia develops in a significant fraction of Spdef<sup>−/−</sup> mice within 18 months in response to antecedent inflammation. We attribute this disorder to defects in maturation of mucous gland cells, which normally express high Spdef levels and in its absence show reduced numbers of secretory granules and reduced expression of Muc6 and Tff2. Thus, in addition to controlling maturation of intestinal goblet and Paneth cells, Spdef also regulates terminal differentiation of antral mucous gland cells. This activity is necessary for proper epithelial barrier function.

**EXPERIMENTAL PROCEDURES**

*Generation of Mutant Mice—Spdef clones were isolated from a 129/SvJ mouse genomic library (Stratagene). The targeting construct contained a 5-kb EcoRI/HindIII fragment and 2.6-kb HindIII/BglII fragment as the left and right homology arms, respectively, flanking a neomycin-resistance (Neo<sup>R</sup>) cassette (see Fig. 2A). For genotyping by Southern analysis, HindIII-digested genomic DNA was hybridized to a 700-bp probe from the 5′ end of the Spdef locus (see Fig. 2B) and SalI-digested DNA with a 500-bp 3′ probe (data not shown). The targeting construct was electroporated into isogenic 11 embryonic stem (ES) cells, and two targeted euploid ES cell clones were injected into C57BL/6 blastocysts. Heterozygote offspring of chimeric males were propagated in a continuous mating scheme on a hybrid 129/SvJ strain background and crossed to produce nullizygous mutants. Mice were handled according to institutional regulations. PCR genotyping used a common reverse primer and specific forward primers to amplify a 300-bp region in the wild-type allele or a 720-bp region spanning Spdef and Neo<sup>R</sup> sequences in the targeted allele (see Fig. 2C, supplemental Table 1). PCR conditions were 94.5 °C for 45 s, 56 °C for 45 s, and 72 °C for 1 min for 35 cycles.*
Gene Expression Analyses—Organs were excised after mouse euthanasia by CO₂ asphyxiation and stored at −80 °C. RNA was isolated using TRIzol reagent (Invitrogen) and treated with DNase I, and first-strand cDNA were synthesized using DNase I, and first-strand cDNA were synthesized using oligo(dT) primers. Quantitative real-time PCR was done using SYBR Green (Roche Applied Science) and a 7500 real-time PCR instrument (Applied Biosystems, Foster City, CA) with 40 cycles of amplification at 95 °C (15 s) and 60 °C (1 min). Primers used for real-time PCR are listed in supplemental Table 1. DNA was extracted from five wild-type and five Spdef⁻/⁻ stomachs and from stomach tissue known to be infected with Helicobacter pylori, using the DNeasy tissue kit (Qiagen, Hilden, Germany). DNA from cultured H. pylori served as an additional positive control.

Transmission Electron Microscopy—Mouse antra were isolated immediately after euthanasia, rinsed in PBS, fixed overnight (2.5% formaldehyde, 5% glutaraldehyde, 0.06% picric acid 0.06% CaCl₂, 0.1 M sodium cacodylate buffer, pH 7.4), post-fixed in OsO₄, and embedded in Epon 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined on a JEOL 1200EX electron microscope at an accelerating voltage of 80 kV.

Quantitation and Statistical Analysis—Real-time RT-PCR results were first normalized to glyceradehyde-3-phosphate dehydrogenase (Gapdh) mRNA levels and subsequently to levels of the corresponding transcript in wild-type littermates. At least 100 antral glands per group were counted for Ki67 staining, expressed as stained nuclei per gland. Secretory granules in mucous gland cells were counted on electron micrographs. Groups were compared using Student’s t test with p < 0.05 regarded as statistically significant.

The abbreviations used are: GS-II, Griffonia simplicifolia lectin II; Ab, antibody.
Expression microarray data quality was determined using the Simpleaffy package of Bioconductor (25), and outliers were checked using the dChip package (26). Arrays with <5% outlier probes were used to identify differential expression. Results were normalized using the Robust Multichip Average algorithm in Bioconductor, based on background correction, normalization, and summarization of signal values (25, 27). Gene expression values were converted from log_{2} to linear scale for class comparison analysis, and transcripts that achieved a mean change >1.3-fold and minimum change >1.2-fold between two groups were considered differentially expressed. An unweighted pair group method with arithmetic mean tree was constructed using a hierarchical clustering technique and Pearson’s correlation as the metric of similarity (28, 29). The expression data matrix was_row-normalized for each gene prior to application of average linkage clustering.

RESULTS

**Spdef Expression in Mouse Stomach**—We mapped Spdef expression by quantitative real-time RT-PCR on RNA isolated from selected wild-type mouse organs. Among these tissues, Spdef expression was highest in the dorsal prostate, as expected (9), followed by the colon and stomach (Fig. 1A). Among the three major stomach compartments, Spdef expression is highest in the antrum, followed by the corpus, and absent in the forestomach (Fig. 1B). To corroborate these findings and to identify Spdef-expressing cell types, we performed immunohistochemistry with a well characterized Ab (17). Nuclear staining, as expected for a transcription factor, was undetectable in the forestomach (supplemental Fig. 1A). In corpus glands, we observed nuclear staining in cells with a clear foamy cytoplasm located in the neck region (Fig. 1C), the known site of mucous neck cells (30). Specific and consistently stronger nuclear staining appeared in similar clear foamy cells at the base of antral glands (Fig. 1D), where mucous gland cells reside. Co-staining of Spdef-stained sections with Alcian blue confirmed expression in antral mucous gland cells (Fig. 1E). We also observed high Spdef expression in Brunner glands, a distinct compartment in the intestinal submucosa at the gastroduodenal junction (supplemental Fig. 1D). The morphology and properties of Brunner glands are similar to those of gastric mucous neck cells (31).

In situ hybridization with a specific Spdef probe confirmed this expression pattern. Although low mRNA levels and background staining of chief (zymogenic) cells in the corpus confounded these efforts (data not shown), we consistently observed a specific signal at the base of antral glands with antisense but not with sense probes (Fig. 1F); riboprobe specificity was verified by the absence of signal in Spdef-deficient antrum (data not shown). Staining of hybridized sections with an Ab against the proliferation marker Ki67 localized Spdef-expressing cells in a distinct compartment of the antral mucosa, below the level of proliferating cells (Fig. 1F). These results together indicate that Spdef marks mucous cells of the neck and the basal region in gastric corpus and antral glands, respectively, with most prominent expression in the antrum.

**Generation of Spdef^{−/−} Mice**—We used homologous recombination in embryonic stem cells to create Spdef-null mice. Our targeting strategy (Fig. 2A) replaces a 5.1-kb fragment encompassing exons 2–5 with a PGK-NeoR cassette. As the replaced exons encode the transactivation domain, the Pointed domain, and most of the Ets DNA-binding domain, the resulting mice...
are predicted to carry a functionally null Spdef allele. RT-PCR analysis confirmed loss of sequences originating in exons 2–3 (supplemental Fig. 1B). We also verified the absence of Spdef by immunostaining (supplemental Fig. 1, C–E) and by in situ hybridization using a probe that corresponds to the deleted exons (data not shown). Heterozygote crosses yielded all three expected genotypes (Fig. 2C). Most Spdef<sup>−/−</sup> mice were viable at birth, although they appeared in ratios slightly lower than expected from heterozygote matings (18% instead of 25%, n = 162, p = 0.16, supplemental Table 3). As discussed below, intestinal gene expression profiles in our Spdef<sup>−/−</sup> mouse strain overlapped considerably with those reported with another, independent null allele (19).

**Polypoid Antral Hyperplasia in the Spdef<sup>−/−</sup> Mice**—Although the stomachs of Spdef<sup>−/−</sup> mice did not differ overtly from those of their wild-type littermates in the first 2 months of life, profound thickening of the antral stomach was evident on gross inspection of a significant fraction of homozygote mutant animals (40.9% at ≥4 months age) at necropsy (Fig. 3A). By histology, mice with such antral thickening showed marked polypoid hyperplasia, with impressive expansion of the epithelium and dilated, elongated, and tortuous foveolae (compare Fig. 3, B and C). Ki67 staining revealed an expanded zone of proliferating antral epithelial cells, located predominantly in the mid-gland (Fig. 3D), although the spectrum of variation included samples with proliferating cells present almost throughout the antral epithelium (supplemental Fig. 2C). The hyperplastic mucosa was separated by myofibroblast bands containing cells that express α-smooth muscle actin (Fig. 3E). Some mutant antra harbored scattered epithelial cysts (supplemental Fig. 2A) or areas with tubular or cribriform growth (supplemental Fig. 2B). No signs of severe dysplasia, carcinoma in situ, or invasive carcinoma were evident, even at 18 months of age (n = 3). Hyperplastic glands expressed the foveolar pit cell marker Muc5ac mainly near the luminal surface (Fig. 3E) and did not stain for the chief cell marker gastric intrinsic factor (Gif), the parietal cell marker Atp4b, or the intestine-specific protein Cdx2 (supplemental Fig. 2, D–F), indicating the absence of tissue heterotopia or metaplasia. No mice showed corpus mucosal abnormalities, including glandular atrophy. In summary, about half of Spdef<sup>−/−</sup> adult mice developed severe polypoid hyperplasia of the gastric antrum over a 1.5-year period of observation.

**Inflammation Precedes Antral Hyperplasia in Spdef<sup>−/−</sup> Mice**—Because antral mucosal hyperplasia occurred in Spdef<sup>−/−</sup> mice with incomplete penetrance and increased in frequency with age, we considered it less likely that the changes were direct effects of Spdef loss and more likely that they represented a secondary response. Acute and chronic gastritis are common causes of reactive and neoplastic stomach mucosal hyperplasia in mice and humans (32, 33), prompting us to assess inflammation in the Spdef<sup>−/−</sup> stomach mucosa. Moderate to severe inflammation was evident in nearly 90% of mutant mice after 4 months in the form of dense leukocytic infiltrates in a band-like pattern among the bases of the antral glands (Fig. 4A). This feature was present even in the absence of epithelial hyperplasia and certainly earlier (Fig. 4A) but absent from wild-type littermates. The characteristic nuclear ring shape permitted recognition of large numbers of neutrophils, and immunostaining confirmed a predominance of myeloperoxidase-expressing granulocytes, with few CD3<sup>+</sup> T or B220<sup>+</sup> B lymphocytes (Fig. 4B), indicating acute inflammation. In cases with accompanying epithelial hyperplasia, typically in older animals, we detected mixed inflammatory infiltrates in the lamina propria with formation of submucosal lymphoid follicles (Fig. 4C). Inflammation was usually absent from the gastric corpus (supplemental Fig. 3A) in the absence of antral hyperplasia but often evident at the corpus-antral junction when antral hyperplasia was present (supplemental Fig. 3B); less dense leukocytic infiltrates were also apparent in the duodenal submucosa in...
Thus, inflammation centered on the gastric antrum, with some rostral and caudal extension. Approximately half of Spdef−/− mice euthanized for analysis between 4 and 18 months showed antral inflammation without hyperplasia, but we never observed antral hyperplasia without accompanying inflammation. We therefore reason that inflammation precedes a reactive hyperplasia of the antral mucosa in Spdef−/− mice. Although Helicobacter infection could have contributed in principle to the gastric antral inflammation, neither histochemistry nor a PCR-based assay for Helicobacter DNA uncovered evidence of Helicobacter infection (supplemental Fig. 3, C and D). Thus, an underlying extrinsic stimulus for antral inflammation is unclear.

Spdef Is Required for Proper Maturation of Mucous Gland Cells—Unlike the gastric corpus, which houses parietal and zymogenic cells, the antral epithelium carries a limited number of cell types (Fig. 1D). We analyzed antra from Spdef−/− and littermate control mice between 1 and 2 months in age, before onset of overt inflammation. Ki67 staining revealed equal proportions of proliferating cells in the mutant and control antra, with normal location of replicating cells above the gland base. Staining for Muc5ac and gastrin indicated normal maturation of foveolar pit and gastrin-producing G cells, respectively (supplemental Fig. 4, A–C). Because Spdef expression is confined to mucous gland cells, we hypothesized that defects in this population may be responsible for the inflammatory prodrome and eventual mucosal hyperplasia in null mice. To test this hypothesis, we first used reverse transcription real-time PCR and found that Muc6 and Tff2 mRNAs, two specific markers of antral mucous gland cells (34, 35), were significantly reduced in Spdef−/− mice (Fig. 5A); Tff2 immunohistochemistry confirmed the result (Fig. 5B). N-Acetyl-d-glucosaminyl moieties on glycoproteins are specifically found in antral mucous gland cells (36), and staining for G. simplicifolia lectin GS-II, which selectively binds this moiety, was also reduced in Spdef−/− antra (Fig. 5C). We characterized the apparent maturation defects further by transmission electron microscopy. Wild-type antral mucous gland cells carried multiple mucus granules in the apex, poised for secretion into the foveolar lumen (Fig. 5D). Granule numbers were significantly reduced in Spdef−/− antra (Fig. 5, D and E) and appeared immature in their frequent lack of the typical composite feature of compartments with high and low electron density (Fig. 5F). Thus, antral mucous gland cells are produced in the absence of Spdef but have significant deficiencies in their secreted products. Ultrastructural analysis further reveals a paucity of secretory granules with a structural defect.
Spdef Regulates a Distinct Set of Stomach Genes—To investigate the transcriptional consequences of Spdef loss, we profiled gene expression in the gastric antrum at 6 weeks of age, when morphologic or inflammatory changes are absent. Fig. 6A shows the 50 genes most significantly deregulated in Spdef−/− antra. Real-time RT-PCR confirmed sample results (Fig. 6B), such as for Thrsp, a gene that is also strongly down-regulated in gastric antral hyperplasia-prone Tff2−/− mice (37). Among 713 genes affected in the antrum, 122 were also dysregulated in the colon, representing a notable overlap (p = 0.0001, Fig. 6C, supplemental Fig. 5). Intestinal expression analysis of a different Spdef−/− strain (supplemental Table 4), suggesting that each mouse line reflects bona fide consequences of Spdef loss. mRNA profiling (Fig. 6A) and RT-PCR analysis (Fig. 6D) of stomach and colon from our Spdef−/− mice confirmed reduced levels of Creb3l4 and Mlph, two genes whose expression correlates most closely with Spdef across multiple tissues (Fig. 6D). Thus, a subset of Spdef-dependent genes, exemplified by such factors, represents an apparently common program in functional maturation of gastric and intestinal secretory cells.

**DISCUSSION**

Distinct regions in the mammalian stomach are distinguished by the presence of highly specialized glandular epithelial cells such as acid-secreting parietal and zymogenic chief cells in the corpus and gastrin-producing endocrine and mucous gland cells in the antrum. In addition to secretory functions, the gastric mucosa also forms a barrier against damage from ingested food and microbes. All epithelial cell lineages derive from a common progenitor, but few transcriptional regulators of cell fate determination and terminal differentiation have been identified. Significant expression of the epithelial Ets protein Spdef in the distal stomach suggested the possibility of such a role.

Spdef is expressed in mucous neck and gland cells of the corpus and antrum, respectively. We show that Spdef gene deletion leads to reduced expression of known protective factors such as Muc6 and Tff2 and to significantly reduced numbers and defects in secretory granules in antral mucous gland cells. These observations parallel recent findings in the intestine, where Spdef specifically regulates maturation of goblet and Paneth cells (19). Taken together with the observation that Spdef controls terminal differentiation of goblet cells in the lung (18) and of luminal epithelial cells in the prostate, this

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transcription factor appears to enable maturation of specialized secretory cells in several organs. Of particular interest in this light are the phenotypic similarities between intestinal goblet and gastric antral mucous gland cells, each of which matures incompletely in the absence of Spdef. These two cell types represent a subset of the highly specialized mucin-producing cells that populate the digestive mucosa and produce acidic mucins necessary to protect the epithelium (38, 39). Needs for particular mucins vary along the gastrointestinal tract, and mucin-producing cells have evolved to meet those needs. Goblet cell numbers increase steadily from the duodenum, where they are sparse, to the colon, where they constitute the majority, and differ materially from the dominant mucinosus cells in the stomach, pit cells, which produce neutral mucins. Spdef-dependent mucous cells in the gastric antrum are distinctive but poorly characterized. Their morphology, location, and staining properties place them closest to the network of submucosal secretory cells in duodenal Brunner glands (31), another poorly characterized population that also shows Spdef expression. The shared dependence on Spdef suggests that although antral mucous and intestinal goblet cells serve different, region-specific functions, they may have evolved from a common cellular ancestor that first used Spdef for coordinate regulation of secretory granule products.

The secretory cell defects did not affect epithelial proliferation or other neighboring cell types in young animals. Over time, however, compromised mucous cell maturation led to antral gastritis in most Spdef−/− mice, with polyposid antral hyperplasia, likely reactive, ensuing in about half the cases. These findings imply that Spdef-mediated mucous gland cell maturation is required for protective barrier function. Spdef−/− mucous gland cells showed reduced expression of Tff2, a protease-resistant peptide that is co-secreted with mucus and thought to help in oligomerization of mucin polysaccharides to form a protective viscid coat (35, 40). Tff2-null mice are prone to epithelial hyperplasia and dysplasia upon *H. pylori* infection, whereas supplementary administration of Tff2 accelerates healing of gastric mucosal ulcers (41). Thus, Spdef may exert protection in part through control of Tff2 expression. We also observed reduced expression of Muc6 and reduced staining with GS-II lectin in antral mucous gland cells, suggesting a broad role for Spdef in completing the maturation necessary to produce an effective epithelial barrier.

Gene expression analysis provided additional clues. Both Spdef−/− antrum and Spdef−/− intestine have reduced levels of Creb3l4, an endoplasmic reticulum-associated transcription factor of unknown function (42). Creb3l4 is the transcript most closely correlated to Spdef across numerous expression datasets, followed by Mlph. *Mlph* is expressed highest in Spdef+ epithelia and belongs to a family that regulates secretory granules (43, 44), suggesting a potential role in the Spdef−/− antral secretory defects. Dmbt1, one of the genes most increased in Spdef−/− antrum, is highly expressed in lung, intestinal, salivary, and mammary gland epithelia and in the neck region of gastric glands; its levels increase in response to inflammation and decrease in terminally differentiated epithelia (45). Thus, increased Dmbt1 may contribute to incomplete mucous cell differentiation in the Spdef−/− antrum. Additional genes with diminished expression in the intestine of an independent Spdef−/− strain, including *Hgfac, Ccl6, Ccl9, Rap1gap*, and *Es1* (19), are also reduced in the antrum, and the colon and antrum share 122 dysregulated genes, indicating that candidate Spdef target genes are common to several types of secretory gut epithelia.

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