Heparanase deglycanation of syndecan-1 is required for binding of the epithelial-restricted prosecretory mitogen lacritin

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Introduction

Cell surface heparan sulfate (HS) proteoglycans are carbohydrate-rich regulators of cell migratory, mitogenic, secretory, and inflammatory activity that bind and present soluble heparin-binding growth factors (e.g., fibroblast growth factor, Wnt, Hh, transforming growth factor β, amphiregulin, and hepatocyte growth factor) to their respective signaling receptors. We demonstrate that the deglycanated core protein of syndecan-1 (SDC1) and not HS chains nor SDC2 or -4, appears to target the epithelial selective prosecretory mitogen lacritin. An important and novel step in this mechanism is that binding necessitates prior partial or complete removal of HS chains by endogenous heparanase. This limits lacritin activity to sites where heparanase appears to predominate, such as sites of exocrine cell migration, secretion, renewal, and inflammation. Binding is mutually specified by lacritin’s C-terminal mitogenic domain and SDC1’s N terminus. Heparanase modification of the latter transforms a widely expressed HS proteoglycan into a highly selective surface-binding protein. This novel example of cell specification through extracellular modification of an HS proteoglycan has broad implications in development, homeostasis, and disease.

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Introduction

Cell surface proteoglycans are key players in epithelial morphogenesis. They form gradients that immobilize mitogens in proximity to signaling receptors (Wang and Laurie, 2004; Häcker et al., 2005; Radtke and Clevers, 2005), contribute to cellular adhesion by ligating the ECM, and at least in one case participate in integrin coupling (Beauvais et al., 2004; McQuade et al., 2006). Cell surface proteoglycans consist of a core protein and associated glycosaminoglycan chains, mainly heparan sulfate (HS). Current dogma states that mitogen, cytokine, and ECM binding is largely the domain of the anionic HS chains (Couchman 2003; Häcker et al., 2005). HS chains are generated by a complement of Golgi polymerases, epimerase, and sulphotransferases during posttranslational modification. Each is thought to vary in relative activity by cell or tissue type (Perrimon and Bernfield, 2000). Thus, within a given epithelium or endothelium, a structurally similar HS chain can be attached to genetically distinct core protein (Zako et al., 2003).

New work has shed light on how HS proteoglycan specificity is generated in development and disease. Most involve extracellular enzymes that affect cell surface HS proteoglycans in unexpected ways. Removal of certain HS 6-O-sulfates by endo-6-O-sulfatases Sulf1 and -2 disrupts the binding of the bone morphogenetic protein inhibitor Noggin, leading to its dispersal and establishment of bone morphogenetic protein signaling (Viviano et al., 2004). In contrast, this same HS modification diminishes FGF binding and assembly with its signaling receptor (Dai et al., 2005). In another extracellular modification mechanism, matrix metalloproteinase-7–dependent shedding of the entire syndecan ectodomain promotes cancer-associated up-regulation of glypican-1 and tumor growth (Ding et al., 2005).

In addition to its HS-dependent signaling mechanisms, recent work has shown that the syndecan core proteins themselves...
Lacritin promotes epithelial proliferation at low nanomolar levels, suggesting a cell surface–binding $K_\text{d}$ in the nanomolar range sufficient for affinity purification of its receptor. An apparent 190-kD cell surface protein eluted from lacritin but not control columns after incubation with detergent lysates of surface biotinylated human salivary gland ductal (HSG) cells in buffer containing physiological levels of NaCl (Fig. 1). Sequencing identified the 190-kD protein as a multimer of human SDC1, a transmembrane proteoglycan that acts as a coreceptor for mitogenic signaling by binding heparin binding growth factors such as FGFs, Wnts, Hhs, and hepatocyte growth factors via its HS glycosaminoglycan chains (Alexander et al., 2002; Capurro et al., 2005); and disrupt carcinoma activity when added as recombinant competitors, presumably by disrupting their assembly with other signaling receptors at the cell surface.

Here, we report on a novel mechanism of syndecan-1 (SDC1) signaling that relies on a direct binding interaction of the extracellular core protein domain of the syndecan and modification of the proteoglycan by HS-modifying enzyme. The mechanism involves the partially characterized prosecretory mitogen lacritin, discovered as a consequence of a search for epithelial differentiation factors (Sanghi et al., 2001). Lacritin is a small (12.3 kD) epithelial-selective human glycoprotein. Lacritin signals to stromal interaction molecule 1, mammalian target of rapamycin, and nuclear factor of activated T cells 1 (NFATC1) via rapid PKC$\alpha$ dephosphorylation and phospholipase D activation (Wang et al., 2006) to potentially regulate differentiation, renewal, and secretion by the nongermative exocrine epithelia that it preferentially targets. With the exception of pancreatic $\beta$-cells (Dor et al., 2004), mechanisms of nongermative epithelial differentiation and renewal are poorly understood. Lacritin-deletion analysis identified a C-terminal mitogenic domain with amphipathic $\alpha$-helical structure (Wang et al., 2006) common to many ligand–receptor or ligand–ligand binding sites (Barden et al., 1997; Siemeister et al., 1998). We report here that lacritin’s C terminus targets the SDC1 core protein as a prerequisite for mitogenesis. A second and novel prerequisite is prior modification or removal of HS from the syndecan by heparanase-1. We postulate that the localized action of heparanase converts a widely expressed cell surface proteoglycan into a localized lacritin-binding protein that is required for mitogenic signaling.
SDC1 band (Fig. 2 A). Lacritin-bound SDC1 was consistently detected in the pellet, implying that the ligation was not solubilized by heparitinase/chondroitinase digestion and therefore may involve the core protein. In keeping with this possibility, lacritin did not target SDC2 or -4 (Fig. 2 C) that share HS chains but only 27–28% ectodomain identity with SDC1. FGF2, as expected, bound all three syndecans via heparitinase-cleavable HS (Fig. 2, B and C).

**SDC1 binding via a lacritin C-terminal domain**

Lacritin truncation analysis recently identified a C-terminal mitogenic domain capable of forming an amphipathic helix as per the receptor-binding domain of parathyroid hormone-like protein (Wang et al., 2006). Could SDC1 binding and mitogenic sites be shared? SDC1 binding was unaffected by deletion (Fig. 3 A) of 5 and 10 amino acids from the C terminus (Fig. 3 B) or 15 and 24 amino acids from the N terminus (Fig. 3 C) of lacritin. However, affinity was substantially diminished after five more C-terminal amino acids were deleted (C-15) and completely abolished from C-25 and C-49 lacritin (Fig. 3 B). These data point to a binding site between amino acids 100 and 109 of mature lacritin that mirrors the mitogenic domain. To validate and further probe this observation, lacritin-SDC1 affinity precipitations were competitively challenged with the truncated lacritin mutants (Fig. 4). Soluble lacritin and N-24, but not C-25 and C-59, inhibited binding. Also inhibitory was recombinant human SDC1 core protein (hS1ED) expressed in *Escherichia coli*, but not HS, CS, or human SDC2 or -4. Collectively, these data suggest that ligation of SDC1 is specified by a region within lacritin’s C terminus that appears to show affinity for SDC1’s core protein but not HS or CS.

**SDC1 is required for lacritin mitogenesis**

Because mitogenic (Wang et al., 2006) and SDC1-binding domains map to the same 10-amino-acid region, we questioned whether competition with recombinant hS1ED would disrupt lacritin-dependent mitogenesis. Soluble hS1ED inhibited proliferation of lacritin-stimulated HSG cells in a dose-dependent manner. The same inhibitory doses had no effect on C-25–treated cells or on FBS-stimulated proliferation (Fig. 5 A). To approach this question differently, HSG cells were depleted of SDC1 by siRNA (Fig. 5 C). Dose-dependent depletion of SDC1, but not depletion of SDC2 (Fig. 5 C), completely abrogated lacritin mitogenic responsiveness (Fig. 5 B). Lacritin signals through Goi or Gαi/PKCα/PLC/Ca2+/calineurin/NFATC1/cyclooxygenase (COX) 2 toward mitogenesis (Wang et al., 2006). We therefore examined COX2 expression in SDC1- and SDC2-depleted cells. In SDC1 but not SDC2 knockdown cells, lacritin-dependent COX2 expression was absent (Fig. 5 D).
Ligation of SDC1 thus appears to be a required upstream step in lacritin mitogenic signaling.

Lacritin and FGF2 target different forms of SDC1

We noted how biotinylated SDC1 from surface-labeled HSG cells was selectively purified on lacritin affinity columns and that it migrated as a relatively distinct band (Fig. 1) without prior heparitinase/chondroitinase to excise the heterogeneous HS and CS chains. In contrast, native SDC1 without digestion presents as a broad smear (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200511134/DC1). Also, lacritin affinity precipitated SDC1 was retained in the pellet after heparitinase/chondroitinase digestion. Could the sharply defined 190- and 80-kD bands represent a minor deglycanated or hypoglycosylated form preferentially enriched because of lacritin’s apparent core protein–related affinity? To explore this possibility, we sequentially depleted either FGF2- or lacritin-bindable SDC1 from lysates and then challenged the depleted lysates with lacritin or FGF2 affinity precipitation, respectively (Fig. 6). Affinity precipitates were treated with heparitinase/chondroitinase before SDS-PAGE to simplify mAb B-B4 detection of the core protein in the digest supernatant (FGF2) or pellet (lacritin). Successive pull-down with FGF2-GST depleted all FGF2-bindable SDC1 (Fig. 6 A, lanes 1–3). Interestingly, the amount of SDC1 available to interact with lacritin-intein was unaffected (Fig. 6 A, lane 4 vs. lanes 1 and 9). Similarly, depletion of SDC1 with lacritin-intein slightly but not substantially diminished SDC1 binding to FGF2-GST (Fig. 6 A, lanes 5–7 vs. lanes 8 and 10). This implies that two pools of SDC1 may be available. One is apparently native SDC1, to which lacritin appears to lack affinity. The other may be an HS-free or partially deglycanated form of SDC1. Could the latter be an immature intracellular form? This appears not to be the case. When cells were gently trypsinized before lysis, no lacritin-bindable SDC1 was detected (Fig. 6 B) in keeping with the original purification of labeled SDC1 from surface biotinylated cells (Fig. 1). Also ruled out was bacterial heparitinase contamination of recombinant lacritin. We took advantage of 3G10 mAb directed against a desaturated uronate epitope generated by heparitinase digestion (David et al., 1992) and could detect lacritin-bound SDC1 only after treatment with exogenous heparitinase (Fig. 6 C). That heparitinase can create the 3G10 epitope is revealing, for it points to the presence of HS or HS stubs on the core protein that is recognized by lacritin. HS stubs could be generated by heparanase, a eukaryotic endo-β-D-glucuronidase that cleaves the entire HS chains between GlcUA and GlcNAc linkages. Collectively, these data suggest that lacritin and FGF2 target different forms of cell surface SDC1. SDC1 bound by lacritin is less heterogeneous, suggesting that although it is decorated with sufficient HS to be recognized by heparitinase, much of its HS has been removed.

Heparanase-dependent lacritin mitogenesis

One hypothesis to explain these data is that heparanase-sensitive HS sterically blocks lacritin binding to a latent core protein site in native SDC1. If this is true, heparanase digestion of native SDC1 should promote lacritin binding (Fig. 7 A). To study this possibility, SDC1 from cell lysates was purified on FGF2-GST, washed, salt eluted, heparanase digested, and incubated with lacritin-intein (Fig. 7 A, lane 1 [0.5 M NaCl eluate] and lane 2 [1.0 M NaCl eluate]). As controls, SDC2 and -4 from cell lysates were individually purified on FGF2-GST, washed, salt eluted, heparanase digested, and incubated with lacritin-intein.

Figure 5. SDC1 is required for lacritin-dependent mitogenesis and COX2 expression. (A) Proliferation assay in which HSG cells were grown for 24 h in serum-free media containing 10 nM lacritin, 10 nM C-25 lacritin, or FBS in the absence or presence of increasing amounts of soluble hS1ED. (B) Identically performed proliferation assay in which HSG cells were treated with 10 nM lacritin or FBS 48 h after being mock transfected or transfected with 10 nM of Ambion’s negative control siRNA #1 (neg), 1–100 nM SDC1 siRNA, or 10 nM SDC2 siRNA. Error bars indicate SEM. (C, top) RTPCR and Western blotting of mock versus 10 nM SDC1 siRNA–treated cells. RT-PCR is for SDC1 and -2 mRNAs. Blotting is with mAb B-B4 for SDC1 core protein or with anti-GAPDH. (bottom) RT-PCR for SDC2 mRNA in mock-transfected cells or cells transfected with 10 nM SCD2 siRNA. (D) RT-PCR of COX2 expression by HSG cells without (+) or with (-) 10 nM lacritin stimulation. 48 h earlier, the cells were mock transfected or transfected with 10 nM SDC1, 10 nM SDC2, or 1 nM heparanase-1 (HPSE-1) siRNAs. At bottom is GAPDH expression.
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(Fig. 7 A, SDC2 [lane 3, 0.5 M NaCl eluate; lane 4, 1.0 M NaCl eluate] and SDC4 [lane 5, 0.5 M NaCl eluate; lane 6, 1.0 M NaCl eluate]). Affinity precipitates were heparitinase/chondroitinase (+) treated before SDS-PAGE and blotting for SDC1 core protein. Shown are digest supernatants (lanes 4–7) as per heparitinase release of FGF2-bound or resistance of lacritin-bound SDC1, respectively. (B) SDC2 (lanes 3 and 4) and SDC4 (lanes 5 and 6) from stably expressing HEK293T cells were individually purified on FGF2-GST, eluted (0.5 and 1 M NaCl), treated with heparitinase I/chondroitinase ABC for 2 h, and incubated with lacritin-intein beads. Blotting is with mAb B-B4 for SDC1 core protein. Lower panels show the blot after treatment with heparitinase I/chondroitinase ABC. (C) Lysates from HEK293T cells stably expressing human SDC1 were incubated with lacritin-intein beads. Beads were washed and treated with heparitinase I/chondroitinase ABC. The digests were centrifuged, and pellets were blotted with mAb 3G10 for desaturated uronates in SDC1.

Figure 7. Bacterial heparitinase digestion exposes FGF2-bindable SDC1 to lacritin binding via a domain in SDC1’s N-terminal 50 amino acids. (A) Human SDC1 (lanes 1 and 2), SDC2 (lanes 3 and 4), and SDC4 (lanes 5 and 6) from stably expressing HEK293T cells were individually purified on FGF2-GST, eluted (0.5 and 1 M NaCl), treated with heparitinase I/chondroitinase ABC for 2 h, and incubated with lacritin-intein beads. Blotting is with mAb B-B4 for SDC1, polyclonal antibody L-18 for SDC2, or polyclonal N-19 for SDC4—all core protein specific. (B) Schematic diagram of human SDC1. The dotted line indicates truncation sites in the ectodomain forming the deletion constructs del 1–51, 51–252, and 51–310. Boxes represent PSIPRED-predicted α helices. Wavy lines represent HS and CS. TM, transmembrane domain. (C) Comparative incubation of FGF2-GST and lacritin-intein beads with human SDC1 or human SDC1 del 1–51 lysates from stably expressing HEK293T cells. After incubation, beads were washed extensively and either treated with heparitinase I/chondroitinase ABC (+) or left untreated (–). Beads were centrifuged, and pellets (P) and supernatants (S) were blotted with mAb 3G10 for desaturated uronates in SDC1. (D) Comparative incubation of lacritin-intein beads with human SDC1 del 1–51, 1–51, or 51–310 lysates from stably or transiently expressing HEK293T cells. pcDNA is lysate from cells transfected with vector only. After incubation, beads were washed extensively and treated with heparitinase I/chondroitinase ABC. Beads were centrifuged, and pellets were blotted with mAb 3G10 for desaturated uronates in SDC1.
50 amino acids as a secreted form (del 51–310; Fig. 7 B). Del 51–252 and 51–310 both bound lacritin, but not del 1–51 (Fig. 7, C and D), suggesting that SDC1’s N terminus is recognized by lacritin.

Although most heparanase is associated with endocytic compartments, the argument for an active cell surface role is compelling. Evidence includes heparanase secretion by activated endothelial (Chen et al., 2004) and T cells during inflammation (Fridman et al., 1987), antisense-inhibited cancer dissemination (Uno et al., 2001), and overexpression-associated migration of hair cell progeny (Zcharia et al., 2005). Is heparanase required for lacritin mitogenic binding of SDC1? Blotting for heparanase-1 detected the active 50-kD form that was enrichable on a HiTrap heparin column from both HSG and HEK293/SDC1 lysates (Fig. 8 A), in keeping with the known affinity of heparanase for heparin. The presence of heparanase in these fractions was confirmed in preliminary activity assays showing digestion of $^{35}$SO4-labeled matrix (unpublished data).

To assess whether heparanase-1 or -2 is required for lacritin-dependent proliferation, we treated HSG cells with siRNAs for each (Fig. 8, B and C). Heparanase-1 is abundantly expressed and, when knocked down, reduced lacritin-dependent proliferation to background in a dose-dependent manner. Importantly, the lowest effective doses did not affect EGF-dependent mitogenesis, and depleted cells were rescued by addition of exogenous heparanase or heparitinase (Fig. 8 C). In depleted cells without lacritin, neither had any effect (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200511134/DC1), thus eliminating the possibility that rescue was instead from heparanase signaling (Gingis-Velitski et al., 2004). Heparanase-2 siRNA also had no effect (Fig. 8 C), but standard RT-PCR failed to detect heparanase-2 expression in untreated cells (not depicted), in keeping with real-time PCR detection of <15 mRNA copies/ng cDNA in human salivary gland (McKenzie et al., 2000).

We noted that lacritin mitogenic signaling promotes COX2 expression downstream of NFATC1 (Wang et al., 2006) and that siRNA depletion of SDC1, but not SDC2, abrogates lacritin-dependent COX2 expression (Fig. 5 D). If heparanase-1 is functionally linked with SDC1 in lacritin mitogenic signaling, then depletion of heparanase-1 should have a similar effect. We observe in Fig. 5 D that this is indeed the case. Lacritin has no effect on COX2 expression in cells lacking heparanase-1.

Thus, it is apparent that two pools of SDC1 are available and that the lacritin-bindable pool is likely generated by heparanase. If this is true, the distribution of HS chain sizes in the FGF2-bindable versus lacritin-bindable pools should differ. To explore this possibility, each pool was isolated by affinity precipitation from $^{35}$SO4-labeled cell lysates. After chondroitinase digestion and elution with NaCl, HS was cleaved from the core protein with NaBH4 and analyzed by CL-6B gel filtration chromatography (Fig. 8 D). In contrast to unimodal HS from the FGF2 pool ($K_{av} = 0.3–0.33; \sim 40$ kD), HS from the lacritin pool was bimodal, with most $^{35}$SO4 eluting with a $K_{av}$ of 0.75–0.8. This corresponds to \sim 4–5 kD. Both estimates are based on Waterson’s standard curve (Waterson, 1971).

Interestingly, lower molecular mass HS was eliminated by heparanase-1 depletion (Fig. 8 D). Collectively, these data suggest
a mechanism whereby SDC1’s HS-rich N terminus is partially deglycanated by heparanase-1 to facilitate lacritin binding (Fig. 9) and signaling to mitogenic COX2.

**Discussion**

How cell surface proteoglycans specify regions of epithelial morphogenesis, homeostasis, or secretion is a central question in developmental biology. We report a new mechanism in which the N-terminal deglycanated core protein of SDC1, and not complete HS/CS chains nor SDC2 or -4, appears to target the epithelial selective proteoceratory mitogen lacritin. An important and novel step in this approach is that binding necessitates prior complete or partial removal of HS chains by endogenous heparanase (Fig. 9). Limiting lacritin activity to specific sites of secreted heparanase thus transforms widely expressed SDC1 into a regulated surface-binding protein.

Recent studies emphasize a growing appreciation for an interaction role of syndecan core proteins beyond the binding accomplished by their HS chains. Sdc1 regulates the activation of the αvß3 and αvß5 integrins in several cell types, an interaction that depends on functional coupling between an extracellular active site in the syndecan core protein and the integrins (Beauvais and Rapraeger, 2003; Beauvais et al., 2004; McQuade et al., 2006). HS plus a short extracellular hydrophobic region near the transmembrane domain of mouse Sdc1 inhibits ARH-77 human B lymphoid cell invasion into collagen I (Langford et al., 2005). Recombinant human SDC2 core protein from *E. coli* mediates adhesion and proliferation of colon carcinoma cells (Park et al., 2002), and mouse Sdc4 contains a high-affinity cell-binding domain proximal to HS attachment sites (McFall and Rapraeger, 1997, 1998). Thus, the ectodomains of syndecan core proteins mediate several morphogenetic and homeostatic events.

Lacritin’s preference for heparanase-deglycanated SDC1 core protein is an interesting cell-targeting strategy that successfully appropriates a ubiquitous proteoglycan for a role as a restrictive cell surface–binding protein. That this is feasible is a reflection of the rarity of SDC1 as a part-time or hypoglycosylated core protein and the lack of general ectodomain sequence conservation among syndecans. Focal heparanase release may regulate lacritin’s mitogenic and proteoceratory activity with unusual accuracy. Focal heparanase degradation of cell surface and ECM HS is implicated in glandular morphogenesis (Zcharia et al., 2004), stem cell migration (Zcharia et al., 2005), and cell survival (Cohen et al., 2006). It also plays a central role in inflammation and cancer (Reiland et al., 2004). Activated endothelial (Chen et al., 2004) and T cells secrete heparanase during inflammation (Fridman et al., 1987). Up-regulation of heparanase mRNA is correlated with reduced HS in invasive esophageal carcinomas (Mikami et al., 2001), whereas the opposite is linked to an increase in overall HS in differentiating myoblasts (Barbosa et al., 2005). Our studies did not address whether SDC2 and -4 are functional targets of heparanase. Neither bound lacritin with or without prior heparitinase treatment. Nonetheless, exploration of other ligands may reveal a similar capacity for latency in these and other HS proteoglycans.

Heparanase-regulated proliferation has previously been attributed to the release of HS-bound FGFs in metastatic breast cancer (Kato et al., 1998). Notably, the first lacritin EST in GenBank derives from a subtracted breast cancer library, and evidence has been presented for lacritin gene amplification in some metastatic breast cancers (Porter et al., 2003). Others have proposed that lacritin is the second most frequent SAGE (serial analysis of gene expression) marker for circulating breast cancer cells (Bosma et al., 2002). Sdc1 is required for Wnt-dependent breast cancer in mice (Alexander et al., 2000) and, in human cancers, is up-regulated in some but not others, coincident with a role in early proliferative events (Ding et al., 2005). Thus, lacritin, heparanase, and SDC1 together potentially offer a new paradigm for some human breast cancers.

Although the sequencing data did not expose lacritin’s putative signaling receptor, use of pharmacological inhibitors and siRNA have identified proximal signaling elements as Gαi or Gαq/PKCo-PLC/Ca²⁺/calcineurin/NFATC1/COX2 and Gαi or Gαq/PKCo-PLC/PLD1/mTOR (Wang et al., 2006). Both are ERK1 and -2 independent and thus contrast with SDC1 cytoskeletal signaling. Lacritin signaling may thus involve a G protein–coupled receptor or G protein–dependent ion channel that gains ligand affinity as a consequence of lacritin immobilization on SDC1. Core protein binding may be stabilized by interaction with HS stubs detected in the lower molecular weight heparanase-dependent peak (Fig. 8 D). Interestingly, because lacritin- and FGF2-bindable SDC1 pools share some HS chains of similar size, not all HS on lacritin-bound SDC1 seem to be cleaved. Lack of complete competition of soluble lacritin for SDC1 in lacritin affinity precipitation assays versus N-24 might hypothetically result from folding of lacritin’s more negatively charged N terminus onto its positively charged C terminus. Cleavage of HS by heparanase to generate lacritin-dependent mitogenic activity offers a novel mechanism of epithelial renewal with important implications to the physiology of human exocrine glands.

Collectively, these observations contribute to the growing appreciation of mechanisms by which extracellular enzymes regulate proteoglycan activity in unexpected ways. Recently described Sulf1 and -2 modify the character of HS chains by selectively removing certain 6-O-sulfate groups, thus altering growth factor signaling and tumor growth (Dai et al., 2005). Heparanase cleavage of HS promotes angiogenesis by solubilizing HS-bound

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**Figure 9. Proposed model of epithelial cell targeting by lacritin.** Deglycanated core protein of SDC1 targets the epithelial selective proteoceratory mitogen lacritin. (i) Binding requires prior partial or complete removal of HS chains by endogenous HPSE1. (ii) Binding is mutually specified by lacritin’s C-terminal mitogenic domain and SDC1’s N terminus.
growth factors (Sanderson et al., 2004). This new discovery that heparanase removal of HS chains removes a block to mitogenic signaling offers a new regulatory paradigm.

Materials and methods

Cell culture, plasmid constructs, and transfection
The HSG cell line was provided by M. Hoffman (National Institute of Dental and Craniofacial Research, Bethesda MD). HSG cells were cultured in DMEM/F12 with 10% FBS. Cells were assayed between passage 10 and 20. For transfection, HSG cells were transfected with a SMARTpool of four human SDC1 (Ambion) or heparanase-1– or heparanase-2–specific siRNAs at different doses (Dharmacon). Other cells were transfected with individual siRNAs also at different doses. siRNAs sequences are as follows: SDC1 siRNAs, GAACAUAAGCGCCAUUGGT, GGAGGAUCCUAUGCGGA, GAACUUCACCCUGAAGACGT, and GGUAGAUAOUAUUGAGT (available from GenBank/EMBL/DDBJ under accession no. NM_002997); SDC2 siRNAs, GGAGUUGUAAUCCGAAACGT, GAAGUAAUGAGCCAG, and GGAGUUAUCCUAGUAUGGT (available from GenBank/EMBL/DDBJ under accession no. NM_002998); heparanase-1 siRNAs, GAAGAAGCCCCAGACAGGUGUU, GAAGAAGCCCCACCGGCAUU, GAAGAGAGGUCCUGGUCAGGU, and GAAGGACAGCAGUCAAGC (available from GenBank/EMBL/DDBJ under accession no. NM_006665). Heparanase-2 siRNA sequences from Dharmacon were not made publicly available. Also used was Ambion’s negative control siRNA #1. Silencing efficiency was evaluated by protein blotting and RTPCR.

HEK293T cells were purchased from and propagated as suggested by American Type Culture Collection. HEK293T cells were transfected with a BglII linearized expression vector coding for human SDC1 (hS1-pcDNA3) using Lipofectamine 2000 reagent (Invitrogen). Stable populations expressing SDC1 were selected in culture medium containing 400 ng/ml G418. A human SDC1 deletion construct lacking 51 amino acids from the N terminus (del 1–51) was generated from hS1-pcDNA3 by long-range reverse PCR using forward primer 5′-GCTGTTGATTACCGGAGTTGTCCTCGACGAGCGCATCCC-3′ and reverse primer 5′-CGCCATGCAGCACAGCACGPOC-3′ containing BamH1 sites. Amplicons were cut using BamH1, ligated as plasmid. Human SDC1 del 51–155 (only N-terminal 50 amino acids of ectodomain linked to the transmembrane and cytoplasmic domains) was similarly generated from hS1-pcDNA3 using forward primer 5′-CTAGCTGAGGAGGACACCGTCCGAGCTG-3′ and reverse primer 5′-CTAAGCTGAGGAGGACACCGTCCGAGCTG-3′. This introduced Nhel sites 5′ of the codon for Ala51 and 3′ of the codon for Glu252 (most C-terminal ectodomain amino acid). Amplicons were digested with Nhel and ligated as plasmid. Human SDC1 del 51–310 (only N-terminal 50 amino acids of ectodomain) was PCR amplified from hS1-pcDNA3 using forward primer 5′-CTAGCTGAGGAGGACACCGTCCGAGCTG-3′ and reverse primer 5′-CTAAGCTGAGGAGGACACCGTCCGAGCTG-3′ containing HindIII and EcoRI sites. Amplicons were digested with HindIII and EcoRI to create cohesive ends and subsequently purified and ligated into the HindIII-EcoRI site of the pcDNA vector. All constructs were confirmed by DNA sequencing. Plasmids were transfected into HEK293T cells, and stable or transient transfectants were generated. Generation of HEK293T cells stably transfected with human SDC2 or -4 was previously described (Utani et al., 2001). Development of lacritin-intein constructs and purification have been described elsewhere (Wang et al., 2006). A lacritin-GST construct was prepared by subcloning lacritin cDNA into pGEX4T-2 (GE Healthcare) using Sap1 and Nde1, in-frame with GST. Recombinant plasmids were transformed into E. coli strain BL21. Bacterial cultures were expanded and fusion protein purified on glutathione–Sepharose 4B (GE Healthcare). A human SDC1 ectodomain-GST construct was generated from pGEX2T hS1ED and similarly purified.

Lacritin affinity chromatography
Cell surface biotinylation and affinity chromatography followed the method of Chen et al. (1997). In brief, six 150-mm culture dishes of 80% confluent HSG cells were washed twice with ice-cold PBS and incubated for 15 min with 50 μl of E2Ulink Sulfo-NHS-Biotin (Pierce Chemical Co.). Cells were then washed twice with PBS-glycine, gently loosened with a cell scraper, and pelleted at 4°C. The pellet was twice resuspended in 25 ml PBS-glycine and incubated for 30 min in 1 ml lysis buffer (50 mM Tris HCl, pH 7.4, 100 mM NaCl, 5 mM MnCl₂, 2 mM PMSF, 200 mM n-octyl-β-D-glucopyranoside, and protease inhibitors [Roche Diagnostics]). Lysate was centrifuged for 15 min at 4°C, and the supernatant was applied to a 1 ml precolumn, washed through with 1 ml binding buffer (50 mM Tris HCl, pH 7.4, 100 mM NaCl, 5 mM MnCl₂, 2 mM PMSF, and 50 mM n-octyl-β-D-glucopyranoside), and collected. Half was applied to a lacritin-intein column, in which lacritin was coupled to chitin beads via chitin-binding intein, and the other half to a negative control column that included an approximately equivalent molar amount of interchin-only. Columns were rotated end-to-end overnight at 4°C, and each was washed with 20 column volumes of affinity column buffer and eluted with the same buffer containing 1 M NaCl. 200 μl fractions were collected per column. Fractions were run on 8% SDS-PAGE gels and silver stained or transferred to nitrocellulose for blotting with streptavidin peroxidase. For the latter, blots were blocked with PBS containing 0.1% Tween 20 and 2.5% milk for 1 h at 37°C, washed three times with PBS/0.1% Tween 20, incubated in 50 ml of 1:1,000 streptavidin-horseradish peroxidase conjugate (GE Healthcare) in PBS/0.1% Tween 20, washed five times with the same buffer, and detected using ECL reagent (Pierce Chemical Co.). Bands of interest were excised and sequenced by mass spectrometry.

Affinity precipitation binding
Human SDC1, -2, or -4 stably expressing HEK293T cells were harvested on ice into 1 ml of the same lysis buffer used for affinity chromatography. Lysates were cleared by centrifugation (20,000 g) at 4°C, and protein concentration of supernatant was estimated by the BCA assay (Pierce Chemical Co.). 5 μg lacritin-intein or lacritin-GST and FGF2-GST fusion proteins were bound to chitin beads (New England Biolabs, Inc.) or glutathione–Sepharose beads, respectively. Beads were incubated with lysates (~200 μg of SDC1 stably expressing HEK293T cells) overnight at 4°C, and washed three times with affinity chromatography binding buffer (each wash three times the bead volume). In competition assays, SDC1 lysates were mixed with increasing amounts of soluble lacritin, HS, HS plus CS (Seikagaku America), bacterially expressed human SDC1 ectodomain (hS1ED), native SDC2, native SDC4, N-24, or C-25. Mixtures were applied to lacritin immobilized beads and further studied. For sequential pull-down assays, cell lysates were sequentially affinity precipitated with FGF2-GST or lacritin-intein. After FGF2-GST depletion of all available FGF-bindable SDC1, one half was precipitated with lacritin-intein. The other half was methanol precipitated overnight and resuspended in heparinase buffer. Similarly, after lacritin-intein depletion of all available lacritin-binding SDC1, one half was precipitated with FGF2-GST and the other half precipitated by methanol overnight and resuspended in heparinase buffer.

The reactions were separated by SDS-PAGE and blotted using anti-SDC1 mAb B-B4 (Serotec) or anti-SDC2 polyclonal antibody L-18 or anti-SDC4 polyclonal antibody N-19 (Santa Cruz Biotechnology, Inc.) followed by ECL detection.

For SDS-PAGE and immunoblotting, beads were digested with heparinase I (Seikagaku America) and chondroitin ABC lyase (MP Biochemicals) because native syndecans migrate as a heterodisperse smear in SDS-PAGE. In brief, beads were resuspended in heparinase buffer (50 mM Heps, pH 6.5, 50 mM NaOAc, 150 mM NaCl, and 5 mM CaCl₂) with 0.0001 U heparitinase and 0.005 U chondroitin ABC lyase for 2 h at 37°C. A second aliquot of each enzyme was added for an additional 2 h. Samples containing each enzyme were precipitated with 20 μl of SDC1 binding beads coated by 10% SDS-PAGE, transferred to Immobilon-P polyvinylidene difluoride (Millipore) for 4 h at 300 mA, fixed for 30 min in PBS containing 0.05% glutaraldehyde (Sigma-Aldrich), and blocked overnight at 4°C in TBS (10 mM Tris and 150 mM NaCl, pH 7.4) with 3% BSA. mAb B-B4 diluted in blocking buffer was incubated with beads for 2 h at RT, washed five times with 10 mM Tris and 150 mM NaCl, pH 7.4, containing 0.1% Tween 20, and detected with alkaline-phosphatase–conjugated secondary antibody (GE Healthcare) using ECL.

Heparanase detection
For analysis, cellular heparanase was enriched by HijTrap heparin affinity purification (GE Healthcare). In brief, HSG or HEK293 cells were lysed overnight against binding buffer (10 mM sodium phosphate, pH 7) and applied to the column. After washing with 10 column volumes of binding buffer, heparanase was eluted using 5 column volumes of elution buffer (10 mM sodium phosphate and 2 mM NaCl, pH 7). Protein concentration was determined by BCA and analyzed by 10% SDS-PAGE. Heparanase was visualized with rabbit polyclonal antibody against human heparanase (provided by I. Vlodavsky, Rappaport Faculty of Medicine, Haifa, Israel) followed by HRP-conjugated secondary antibody and ECL.

Mitogen assay
HSG cells in serum-containing media were seeded in 24-well plates at a density of 0.5 × 10⁵ cells/well. After 24 h, the medium was changed to minimum essential medium alpha modification with washes for 24 h, and
lacritin was added for 24 h to a final concentration of 10 nM in the same medium containing 2 μCi/ml [3H]-thymidine. Cells were incubated alone with lacritin or together with an increasing amount of bacterial-expressed human SDC1 ectodomain (hS1ED) as a soluble inhibitor. Cells depleted of heparanase-1 or SDC1 were treated with lacritin in [3H]-thymidine 48 h after siRNA transfection. To rescue heparanase-depleted cells, ~1 μg heparanase enriched from HSG or HEK293 cells using heparin affinity column after siRNA transfection. To rescue heparanase-depleted cells, [3H]-thymidine incorporation was stopped by placing on ice. Cultures were washed twice with ice-cold PBS, fixed with cold and RT TCA (10%) for 10 min each, washed twice with RT PBS, collected in 1 N NaOH, neutralized with 1 N HCl, and transferred to liquid scintillation vials for measurement.

HS chain analysis
50% confluent HSG cell cultures in 150-mm culture dishes were metabolically labeled with 50 μCi/ml NaH2[3]H4SO4 (1494 Ci/mmol; PerkinElmer) in DME for 48 h as described by Zako et al. (2003). Both normal and heparanase-1-depleted cells were labeled. After washing three times with PBS, cells lysates were collected and affinity precipitated with FG2-GST or lacritin-tein overnight at 4°C. SDC1 bound to beads was digested with chondroitin ABC lyase [MP Biochemicals] for 3 h at 37°C, eluted with 2 M NaCl, and subjected to eliminative cleavage and reduction of HS by adjusting to 100 mM NaOH/1 M NaBH4 for 24 h at 37°C. Released HS was neutralized by drop-wise addition of 1 M HCl and subjected to Sepharose CL-6B column (1 × 57 cm) gel filtration chromatography in PBS at a flow rate of 16 ml/h. Radioactivity was measured by liquid scintillation counting. The void volume (V0, fraction 26) and total column volume (V, fraction 62) were determined using dextran blue and sodium dichromate, respectively, as markers.

Online supplemental material
Fig. S1 displays the size heterogeneity of native SDC1 attributable to its HS and CS chains. Fig. S2 demonstrates that heparanase and heparanase-1 alone are not mitogenic for HSG cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200511134/DC1.

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