Growth factor–induced shedding of syndecan-1 confers glypican-1 dependence on mitogenic responses of cancer cells

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The cell surface heparan sulfate proteoglycan (HSPG) glypican-1 is up-regulated by pancreatic and breast cancer cells, and its removal renders such cells insensitive to many growth factors. We sought to explain why the cell surface HSPG syndecan-1, which is also up-regulated by these cells and is a known growth factor coreceptor, does not compensate for glypican-1 loss. We show that the initial responses of these cells to the growth factor FGF2 are not glypican dependent, but they become so over time as FGF2 induces shedding of syndecan-1. Manipulations that retain syndecan-1 on the cell surface make long-term FGF2 responses glypican independent, whereas those that trigger syndecan-1 shedding make initial FGF2 responses glypican dependent. We further show that syndecan-1 shedding is mediated by matrix metalloproteinase-7 (MMP7), which, being anchored to cells by HSPGs, also causes its own release in a complex with syndecan-1 ectodomains. These results support a specific role for shed syndecan-1 or MMP7–syndecan-1 complexes in tumor progression and add to accumulating evidence that syndecans and glypicans have nonequivalent functions in vivo.

Introduction

Many growth factors use heparan sulfate proteoglycans (HSPGs) as cofactors in receptor binding and/or signaling. Dependence on HSPGs has been demonstrated for FGFs, heparin-binding members of the EGF family, such as heparin-binding EGF-like growth factor (HB-EGF) and the heregulins, hepatocyte growth factor (HGF), Wnts, hedgehogs, and at least some members of the transforming growth factor β superfamily (Rapraeger et al., 1991; Aviezer and Yayon, 1994; Zioncheck et al., 1995; Bellaiche et al., 1998; Tsuda et al., 1999; Li and Loeb, 2001; Fujise et al., 2003).

The major HSPGs of the cell surface are the syndecans and glypicans (Lander and Selleck, 2000; Perrimon and Bernefield, 2000). The syndecans are four related transmembrane proteins that sometimes also carry chondroitin sulfate. The glypicans are six glycosylphosphatidylinositol (GPI)-anchored proteins that exclusively carry heparan sulfate. We previously reported that the expression of glypican-1 (but not other glypicans) is induced in human pancreatic and breast cancer cells and that the ability of heparin-binding growth factors to drive the proliferation of these cells is blocked by phosphoinositide-specific PLC (PIPLC), an enzyme that releases GPI-anchored proteins from the cell surface (Kleeff et al., 1998; Matsuda et al., 2001). Responsiveness can be restored with a transmembrane variant of glypican-1 (i.e., one that cannot be cleaved by PIPLC). Conversely, the loss of proliferative response results when antisense RNA is used to decrease levels of endogenous glypican-1. Manipulation of glypican-1 levels with either PIPLC or antisense RNA affects mitogenic responses to growth factors that are HSPG dependent, such as FGF2 and HB-EGF, but not other growth factors such as insulin-like growth factor-1 and EGF (Kleeff et al., 1998; Matsuda et al., 2001). Inhibition of glypican-1 expression also causes pancreatic carcinoma cell lines to form tumors that grow more slowly in vivo (Kleeff et al., 1999).

These studies suggest that glypican-1 plays an important role in the development of at least some cancers. Such a strong dependence on a glypican is surprising given that most cells have both glypicans and syndecans and that both HSPG families function efficiently as growth factor coreceptors (Steinfeld et al., 1996; Zhang et al., 2001). Indeed, substantial syndecan-1 is made by both the pancreatic and breast cancer cells that are dependent on glypican-1 for their growth factor responses (Conejo et al., 2000; Matsuda et al., 2001). These data suggest
that, in some circumstances at least, glypicans and syndecans do not function equivalently. In this study, we take up the question of why this is.

Results

Previous studies uncovered a requirement for glypican-1 in the responses of pancreatic and breast cancer cells to several polypeptide mitogens, including FGF2, HB-EGF, HGF, heregulin-α, and heregulin-β (Kleeff et al., 1998, 1999; Matsuda et al., 2001). In those studies, mitogenesis was quantified as an increased cell number 48 h after growth factor addition. As this endpoint is far downstream of initial growth factor signaling, we tested for glypican dependence at earlier times. Fig. 1 A shows that when PANC-1 pancreatic carcinoma cells are treated with FGF2, the increase in incorporation of [3H]thymidine during the first 24 h is dramatically reduced by pretreatment with PIPLC, just as it is by pretreatment with heparinase III (which removes heparan sulfate from the cell surface). This effect of PIPLC was completely rescued by the expression of a transmembrane glypican-1 variant (Fig. 1 B).

The inhibitory effect of PIPLC on the FGF2 response was also evident at the level of MAPK activation and there, too, could be rescued by a transmembrane glypican-1 (Fig. 1, C and D). In these experiments, MAPK induction was measured as the level of phosphorylation of p42/44ERK 1 h after FGF exposure.

The ability of HSPGs to act as FGF coreceptors is thought to be a function of heparan sulfate chains and independent of core protein structure. The fact that PANC-1 cells express syndecan-1 (Conejo et al., 2000) but PIPLC (which removes only GPI-anchored molecules) blocks the responsiveness to FGF2 strongly suggests that the syndecan-1 on these cells is not an FGF coreceptor. Trivial explanations for this could be that there is not enough syndecan-1, it is not localized to the cell surface, or it lacks heparan sulfate. A variety of observations argue against these possibilities, the most general of which is shown in Fig. 2. Cells were cultured in the presence of [35S]sulfate, and the release of sulfated glycosaminoglycans (GAGs) was measured in response to either PIPLC or to a mild trypsin treatment that selectively cleaves cell surface syndecan-1, which has a juxtamembrane protease-sensitive site, to release an intact ectodomain (Subramanian et al., 1997). Released GAG-containing polypeptides were digested and precipitated to separately quantify protein-bound heparan and chondroitin sulfates.

The results (Fig. 2) show that the amount of heparan sulfate released by PIPLC is approximately the same as that released by mild trypsinization. Moreover, after PIPLC treatment, trypsin released almost as much heparan sulfate as from cells that were not PIPLC treated (Fig. 2, second and third set of bars). Thus, PIPLC and trypsin release nearly nonoverlapping pools of cell surface heparan sulfate, with PIPLC-resistant heparan sulfate making up at least half of the total. From this, we conclude that on the surface of PANC-1 cells, HSPGs that are not glypicans and that possess the trypsin sensitivity of syndecan-1 are at least as abundant a carrier of heparan sulfate as are glypicans.

Although these results raised the possibility that differences in the structure of heparan sulfate on syndecan-1 versus glypican-1 might explain their differential use as FGF coreceptors, an alternate explanation came to mind after we examined the responses of PANC-1 cells to very brief FGF2 exposures.
because the pool of trypsin-released syndecan-1 probably in-
substantial reduction in syndecan-1 remaining on cell surfaces.

As shown in Fig. 4 A, FGF2 caused a ever syndecan-1 remained on the cells; these were then quanti-
and then used mild trypsin to release the ectodomains of what-
de can-1 was not seen (Subramanian et al., 1997).

We also exposed cells to FGF2 for 30 min, washed, and reexposed to FGF2 for 15 min, MAPK activation was substantially lower in the PIPLC-treated cells (Fig. 3 B). PIPLC blocks long-term but not short-term MAPK activation. (A) Where indicated, PANC-1 cells were incubated with 1 U/ml PIPLC for 1 h. Serum-free medium containing 1 ng/ml FGF2 (with 1 U/ml PIPLC where used) was added, and incubation continued for either 15 min or 1 h. Cells were lysed, and activated MAPK (p42/44ERK) was detected by immu
 blotting. As a control for protein loading, immunoblotting was performed with an anti-β-tubulin mAb. Results from duplicate cultures are shown. (B) Cells were treated with FGF2 and analyzed as in A except that some samples were also exposed to FGF2 during the 15 min before the 1-h PIPLC incubation. When such cells were subsequently reexposed to FGF, short-term (15 min) MAPK activation was substantially PIPLC sensitive (asterisk; P < 0.02; t test). Data are duplicates ± SEM (error bars). Y axis is measured in arbitrary units.

PIPLC had no significant effect (Fig. 3 A). However, if cells were first exposed to FGF2, treated with or without PIPLC for 1 h, and reexposed to FGF2 for 15 min, MAPK activation was substantially lower in the PIPLC-treated cells (Fig. 3 B).

These results suggest that when cells are first exposed to FGF2, glypican-1 is not the only HSPG that can serve as an FGF coreceptor, but FGF2 triggers an event that causes it to become so later on. An obvious candidate for such an event would be loss of syndecan-1 from the cell surface. Indeed, syndecan-1 (as well as other syndecans) is known to be shed from cell surfaces through cleavage by endogenous proteases. Moreover, a variety of ligands can trigger such shedding, including EGF and HB-EGF (Subramanian et al., 1997; Fitzgerald et al., 2000), although in the only case in which FGF2 was examined (a lymph node endothelial cell line), induced shedding of syndecan-1 was not seen (Subramanian et al., 1997).

To test whether FGF2 might cause PANC-1 cells to shed their syndecan, we exposed cells to FGF2 for 30 min, washed, and then used mild trypsin to release the ectodomains of whatever syndecan-1 remained on the cells; these were then quantified by immunoblotting. As shown in Fig. 4 A, FGF2 caused a substantial reduction in syndecan-1 remaining on cell surfaces. In three independent experiments, we observed a mean decrease of 59 ± 11%. This value is likely to be an underestimate because the pool of trypsin-released syndecan-1 probably in-
cludes some molecules derived from dead cells, cell fragments, or substratum-attached material that would not be expected to respond to FGF2.

To test whether shedding of syndecan-1 plays a causal role in its failure to act as a long-term FGF coreceptor, we transected PANC-1 cells with a mouse syndecan-1 in which the juxtamembrane protease-sensitive site had been replaced with a heterologous sequence. This form is known to resist shedding by endogenous proteases (Fitzgerald et al., 2000). As a control, we used wild-type mouse syndecan-1. As shown in Fig. 4 B, substantial expression of both wild-type and cleavage site–modified syndecan-1 could be detected in stably transfected cell clones. As was the case with the endogenous syndecan-1, we could readily detect FGF2-induced shedding of exogenous wild-
type mouse syndecan-1 but saw no evidence for shedding of the cleavage mutant form (Fig. 4 C). When cells expressing these constructs were tested for MAPK activation 1 h after FGF2 addition, cells expressing cleavage-resistant syndecan-1 showed no inhibition by PIPLC, whereas the responses of cells expressing wild-type syndecan-1, like sham-transfected cells, were highly PIPLC sensitive (Fig. 4 D). Thus, when cells express a form of syndecan-1 that cannot be shed, long-term responses to FGF2 lose their glypican dependence.

Studies in rodent cells suggest that the enzymes responsible for syndecan-1 shedding are members of the matrix metalloproteinase (MMP) family (Subramanian et al., 1997;
FGF2-induced shedding of syndecan-1, and an "unsheddable" syndecan-1 makes FGF2 responses PIPLC resistant. (A) PANC-1 cells were treated with or without 2 ng/ml FGF2 for 30 min. After washing, trypsin was used to specifically release syndecan-1 ectodomains. Half of each sample was digested with 8 μM/ml heparinase III and 0.1 U/ml chondroitinase ABC. Syndecan-1 ectodomains were detected by Western blotting with mAb B-B4. Exposure times for the first and second lanes were longer than for the third and fourth lanes. (B) PANC-1 cells were stably transfected with expression constructs for wild-type mouse syndecan-1, an engineered variant of mouse syndecan-1 that replaces the cleavage sequence required for shedding with a heterologous one, or empty expression vector. Multiple clones of each type were expanded and examined by immunocytochemistry (not depicted) and Western blotting using mouse-specific syndecan-1 mAb 281.2. Western blot results from three representative clones are shown. Lanes 1 and 2, sham transfected; lanes 3 and 4, wild-type syndecan-1; lanes 5 and 6, cleavage mutant syndecan-1. (C) PANC-1 cells stably transfected with wild-type or cleavage mutant mouse syndecan-1 (from B) were treated with 2 ng/ml FGF2 for 30 min. After rinsing, cells were treated with trypsin as in A, and the released material was digested with 8 μM/ml heparinase III and 0.1 U/ml chondroitinase ABC. Syndecan-1 core protein was measured as in B. (A–C) Arrowheads show positions of molecular mass standards (in kD). (D) The three clones shown in B were tested for MAPK activation 1 h after the addition of 1 ng/ml FGF2. For both the sham-transfected and wild-type syndecan-1–transfected clones, pretreatment with 1 U/ml PIPLC for 1 h dramatically reduced FGF signaling (P < 0.02 in both cases; asterisks), whereas in the clone-expressing cleavage mutant syndecan-1, no significant reduction was seen. Data are from triplicate cultures for each condition and are normalized to loading controls. Error bars represent SEM. Y axis is measured in arbitrary units.

Figure 5. Inhibition of metalloproteinases protects cells from PIPLC inhibition of the FGF2 response. (A) Metalloproteinase inhibitors block FGF2-induced shedding of syndecan-1. PANC-1 cells were treated with or without 1μM GM6001 or 500 ng/ml TIMP-3 for 1 h and with 2 ng/ml FGF2 for 30 min. Syndecan-1 remaining on cell surfaces was released with trypsin, concentrated, digested with heparinase and chondroitinase, and quantified by Western blotting with mAb B-B4 as in Fig. 4. Arrows show positions of molecular mass markers. (B) Metalloproteinase inhibition makes long-term FGF2 responses of tumor cells PIPLC insensitive, whereas the FGF2 responses of a nontumor cell line are already insensitive to PIPLC. PANC-1 cells, MDA-MB-468 breast carcinoma cells, and C2C12 mouse myoblasts were treated for 1 h with 1 μM GM6001, 500 ng/ml TIMP-3, or no protease inhibitor as indicated. Cells were then cultured for 1 h in the presence of 1 ng/ml FGF2 or no growth factor (control). FGF2 + PIPLC cells were also exposed to 1 U/ml PIPLC during both the first and second hours of incubation. Cell lysates were probed for p42/44MAPK activation as in Figs. 1–4.
FGF2 signaling was not itself inhibited by the heparin. FGF2, and tested for the release of syndecan-1. As shown in Fig. treated PANC-1 cells with heparin, washed, exposed the cells to membrane proteins. Accordingly, MMP7—but not MT-MMPs Woessner, 2000), whereas MT-MMPs and ADAMs are integral it associates with cell surfaces through binding to GAGs (Yu and syndecan-1 shedding enzymes are MT1-MMP, MT3-MMP, MMP7 is solely responsible for syndecan-1 shedding. Other pro- and active MMP7 on the surface of untreated cells and found that the levels of both forms were lower after FGF2 treatment (Fig. 6 C). In contrast, when we examined what was released by cells during growth factor exposure, we saw more MMP7, especially the active form, in the material released from FGF2-treated as opposed to untreated cells (Fig. 6 C). These results suggest that FGF2 not only activates MMP7 but that it causes newly activated MMP7 and some pro-MMP7 to be released from the cell surface. This makes sense if one recalls that cell surface MMP7 is associated with heparan sulfate, that about half of the heparan sulfate on PANC-1 cells exhibits the protease sensitivity of a syndecan (Fig. 2), and that MMP7 cleaves syndecan-1. Newly activated MMP7 molecules would thus be expected to induce their own shedding in association with syndecan-1 ectodomains. We can show that this occurs by collecting the material released by FGF2-treated PANC-1 cells, immunoprecipitating syndecan-1, and probing the immunoprecipitate with antibody to activated MMP7 (Fig. 6 D). The results confirm that MMP7–syndecan-1 ectodomain complexes are specifically released when PANC-1 cells are treated with FGF2.

Although these results demonstrate that FGF2 is sufficient to activate MMP7 and that activated MMP7 is sufficient to account for both the shedding of syndecan-1 and the subsequent glypicancan dependence of cell growth, they do not prove that MMP7 is solely responsible for syndecan-1 shedding. Other proposed syndecan-1 shedding enzymes are MT1-MMP, MT3-MMP (Endo et al., 2003), and ADAM metalloproteinases (Holen et al., 2001). One distinguishing feature of MMP7 is that it associates with cell surfaces through binding to GAGs (Yu and Woessner, 2000), whereas MT-MMPs and ADAMs are integral membrane proteins. Accordingly, MMP7—but not MT-MMPs or ADAMs—can be released by heparin. Therefore, we pre-treated PANC-1 cells with heparin, washed, exposed the cells to FGF2, and tested for the release of syndecan-1. As shown in Fig. 7 A, heparin pretreatment blocked the ability of cells to shed syndecan-1. FGF2 signaling was not itself inhibited by the heparin pretreatment; in fact, such signaling became PIPLC insensitive (i.e., glypicancan-1 independent), as would be expected if syndecan-1 were remaining on the cell surface (Fig. 7 B). These data argue that the molecule responsible for syndecan-1 shedding and glypicancan-1-dependent mitogenesis is heparin displaceable, as would be expected for MMP7.

To establish definitively that this molecule is MMP7, we took advantage of the fact that a mAb specific for mature MMP7 has been shown to be function blocking (Wroblewski et al., 2003). As shown in Fig. 7 C, pretreatment of PANC-1 cells with this antibody abrogated the ability of PIPLC to inhibit the long-term FGF2 response, whereas pretreatment with control antibody had no effect.
The mechanisms by which growth factors activate pro-MMP7 are diverse and still poorly understood, although they all ultimately result in cleavage of a propeptide (Crabbe et al., 1992). Interestingly, when one treats PANC-1 cells with FGF2 in the presence of GM6001, one can see an increase in the level of activated MMP7 (using an antibody specific for this form of the enzyme) on the cell surface (Fig. 7 D). It makes sense that the newly activated MMP7 remains on the cell surface as opposed to being shed (Fig. 6 C) given that GM6001 should prevent syndecan-tethered MMP7 from re-association with GPI-anchored proteins and by itself will render initial responses to FGF2, substantial mature (activated) MMP7 is detected on PANC-1 cells presenting a great deal of activation in increased MMP7 (P < 0.05; t test). (C and D) Y axis is measured in arbitrary units.

The aforementioned results argue that in PANC-1 pancreatic carcinoma cells, both glypican-1 and syndecan-1 can act as coreceptors for FGF2, but because FGF2 activates MMP7, which, in turn, induces the shedding of syndecan-1, a sustained mitogenic response requires glypican-1. This conclusion is based on several observations. The FGF2 response of these cells is not initially sensitive to the removal of GPI-anchored proteins by PIPLC but becomes so after exposure to FGF2. FGF2 induces syndecan-1 shedding and also induces the activation of MMP7. Active MMP7 causes syndecan-1 shedding and by itself will render initial responses to FGF2 glypicanc dependent. When syndecan shedding is blocked with GM6001, TIMP-3, expression of a cleavage-resistant syndecan-1, depletes cell surface MMP7 with heparin, or immunological blockade of MMP7, FGF2 responses no longer become glypicanc dependent.

Although this study focuses on the FGF2 responses of pancreatic carcinoma cells, it is likely that the phenomenon described here has wider relevance. First, glypicanc dependence is observed in the growth factor responses of breast cancer cells, and it is abrogated by the metalloproteinase inhibitor GM6001 (Fig. 5 D). Second, long-term responses to multiple heparin-binding growth factors (e.g., HB-EGF, HGF, and heregulins) are glypican dependent in pancreatic and breast cancer cells, and at least one of these factors (HB-EGF) is known to induce syndecan-1 shedding in some cells (Subramanian et al., 1997). Third, all four syndecans (not just syndecan-1) undergo shedding, with metalloproteinase dependence established for the shedding of syndecans-3 and -4 (Subramanian et al., 1997; Asundi et al., 2003). Indeed, although syndecan-1 is the major syndecan on PANC-1 cells, we can detect in these cells small amounts of mRNA for syndecans-2, -3, and -4 (unpublished data), raising the possibility that shedding of these syndecans could be of quantitative significance.

It is interesting that even before exposure to FGF2, substantial mature (activated) MMP7 is detected on PANC-1 cells (Fig. 6 C). The fact that substantial syndecan-1 is also present on the cell surface suggests that most of this MMP7 is nonfunctional, which is presumably a result of complex formation with an endogenous inhibitor (e.g., TIMP-1, which pancreatic carcinoma cells frequently overexpress; Zhou et al., 1998). Accordingly, when pro-MMP7 molecules are activated by exposure to FGF2, one might expect them to eventually become inhibited too. In agreement with this prediction, when we treat PANC-1 cells with FGF2 in the presence of GM6001 (Fig. 7 D) so that newly activated MMP7 is reversibly blocked and then remove GM6001 after 1 h, we do not observe an onset of syndecan-1 shedding (unpublished data). This suggests that over the course of an hour, activated MMP7 molecules do become stably inactivated.

It is provocative that the one nontransformed cell type studied here (C2C12 myoblasts) showed no evidence for glypicanc dependence of long-term FGF2 signaling, suggesting that growth factor–mediated syndecan shedding may be a phenomenon that is more common in tumor cells than in normal cells.
If so, it increases the likelihood that the inhibition of glypican function could be of therapeutic value in selectively inhibiting tumor cell growth.

Our results add to an expanding list of studies on the functions of cell surface HSPGs (Bernfield et al., 1999; Lander and Selleck, 2000; Park et al., 2000; Perrimon and Bernfield, 2000). The degree to which syndecans and glypicans have similar versus different functions is a long-standing question. Comparisons among heparan sulfate structures on glypicans and syndecans on the same cells have failed to identify functionally relevant differences (Liu et al., 1996; Tumova et al., 2000; Zako et al., 2003), yet in a variety of in vitro systems, syndecans have been seen to carry out functions that glypicans cannot. For example, syndecan-1, but not glypcan-1, inhibits the invasive behavior of myeloma cells, which is a function that maps to parts of the syndecan-1 core protein (Liu et al., 1996; Langford et al., 2005). In contrast, there are few direct examples of glypicans performing functions that syndecans cannot. Recently, one group reported that glypicans, but not syndecans, can support growth factor responses of glioma-associated brain endothelial cells (Qiao et al., 2003). It will be interesting to see whether, as in this study, such specificity arises as a consequence of induced syndecan shedding and, if so, whether MMP7 (which is not commonly found at high levels in gliomas; Vince et al., 1999) or a different protease is the culprit.

From a more general perspective, it is remarkable that the in vivo effects of the loss of function of glypicans in man, mice, frogs, and flies almost universally are abnormalities in growth or growth factor signaling (Pilia et al., 1996; Jackson et al., 1997; Tsuda et al., 1999; Grisaru et al., 2001; Desbordes and Sanson, 2003; Galli et al., 2003), whereas syndecan loss-of-function mutations influence cell adhesion, migration, axon guidance, neuropeptide activities, and synaptic function (Woods and Couchman, 2001; Bellin et al., 2002; Bhanot and Nussenzweig, 2002; Ishiguro et al., 2002; Kaksonen et al., 2002; Reizes et al., 2003; Steigemann et al., 2004) but only rarely influence cell growth (Alexander et al., 2000). As more genetic studies are undertaken, it will be interesting to see the extent to which this dichotomy holds up and whether growth factor–induced syndecan shedding explains some or all of it.

This study raises a number of questions concerning the roles of HSPGs in tumor formation and progression. Although it is widely accepted that overexpression of growth factors and their receptors by cancer cells plays a pivotal role in tumor progression, mendelsohn and baselga, 2000; friess et al., 1996; mendelsohn and baselga, 2000; haddad et al., 2001; leroth and roberts, 2003; yu et al., 2003), the up-regulation of HSPG coreceptors by at least some tumor cells has only recently been appreciated (kleeff et al., 1998; matsuda et al., 2001; zhu et al., 2001; nakatsura et al., 2004). In part, this is because most early studies of HSPGs in cancer focused on syndecan-1 and generally reported no increase or even a decrease in its expression (nackaerts et al., 1997; pulkkinen et al., 1997; fujiimoto and kohto, 1998; wikensten et al., 2000, 2001; harada et al., 2003). However, in pancreatic carcinoma cells, both syndecan-1 and glypican-1 are highly up-regulated (kleeff et al., 1998; conejo et al., 2000; barbareschi et al., 2003). In view of the present data, it seems likely that whatever advantage is afforded such cells by expressing syndecan-1, it comes from the shed, not the cell surface, form of the molecule. Interestingly, yang et al. (2002) reported that the growth of human myeloma cells in vivo is strongly enhanced when the cells are engineered to express a constitutively shed form of syndecan-1. How shed syndecan-1 enhances tumor growth is unclear, but an attractive hypothesis is that syndecan ectodomains influence the extracellular proteolysis that is essential for tumor invasion and metastasis. Kainulainen et al. (1998) found that syndecan-1 ectodomains enhance proteolytic activities in wound fluids by protecting proteases from their endogenous inhibitors. Moreover, the activity of MMP7, which is strongly implicated in tumor progression (Yamamoto et al., 2001) and metastasis (Wilson et al., 1997), is stimulated by heparin (Yu and wessner, 2000) and, therefore, may be greater when MMP7 is complexed with syndecan-1.

Another intriguing possibility, suggested by the present data, is that syndecan-1 shedding aids in transporting activated MMP7 away from tumor cells and into surrounding stroma. Ordinarily, one might expect that MMP7, which so strongly binds heparan sulfate, would not readily diffuse away from tumor cells as a result of trapping by cell surface and extracellular matrix HSPGs. By associating with syndecan-1 ectodomains (fig. 6 D), MMP7 could avoid such trapping and, thereby, act at a greater distance. Such a mechanism of action is strikingly analogous to that demonstrated by li et al. (2002) for syndecan-1 ectodomains as promoters of the diffusion of chemokines away from injured lung epithelial cells, an essential step in leukocyte recruitment.

Materials and methods

Materials

Materials were purchased from the following companies: DME and Leibovitz L-15 medium (MediaTech, Inc.); OptiMEM (Invitrogen); HBSS (Invitrogen Scientific); FBS (Hyclone); penicillin–streptomycin solution and l-glutamine (Invitrogen); LipofectAMINE and Geneticin (G418; Gibco BRL); methyl[3H]thymidine and [35S]sulfate (PerkinElmer); detergent of Syndecan Sheding and Glypican Dependence of Growth • Ding et al. 735
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**Cell culture and transfection**

PANC-1 cells were cultured in DMEM with 6% (vol/vol) FBS. MDA-MB-468 breast carcinoma cells were cultured in L-15 medium with 10% (vol/vol) FBS. C2C12 myoblasts were cultured in DMEM with 15% FBS. Antibiotics (100 U/ml penicillin G and 100 μg/ml streptomycin sulfate) were added to all media. Cells were maintained at 37°C in a 5% CO2 atmosphere. Stable transfection of full-length mouse syndecan-1, cleavage-resistant mouse syndecan-1 (Fitzgerald et al., 2000), and pCDNA3.1/glyp1-VS-VGTM (Kleeff et al., 1998) into PANC-1 cells was performed using LipofectAMINE (Kleeff et al., 1998). After reaching confluence, cells were split into complete medium with 1 mg/ml G418. 2–3 wk later, independent colonies were isolated. The expression of glyp1-VSGTM was evaluated by immunostaining using anti-mouse antibodies. The expression of syndecan-1 was evaluated by immunostaining using anti–mouse syndecan-1 mAb 281.2 and by immunoblotting of detergent extractable proteoglycans. For the latter measurements, cells were extracted with 2% Triton X-100, 0.15 M NaCl, 10 mM EDTA, 10 mM KH2PO4, pH 7.5, along with 5 μg/ml BSA, 100 μg/ml PMSF, and 25 μg/ml Nethymole-mide (NEM). After centrifugation, supernatants were subjected to DEAE-Sephacel purification and were eluted with 150, 250, and 750 mM NaCl. FBS. C2C12 myoblasts were cultured in DME with 15% FBS. Antibiotics (containing fresh heparinase III and/or PIPLC, where used), and incubation continued for 24 h. Monolayers were washed with PBS, fixed with methanol (50% in 50 mM Tris-HCl, pH 8.0, at 4°C). The pool eluted by 750 mM NaCl was desalted on PD-10 followed by lyophilization. The resulting material was digested with 10 μl/ml heparinase III (37°C overnight in 3 mM Ca(OAc)2, 50 mM NaOAc, 50 mM HEPES, and 10 mM EDTA, pH 6.5) and chondroitinase ABC (20 μl/ml in 0.1 M Tris-HCl, 10 mM EDTA, pH 7.5) and subjected to 4–20% gradient SDS-PAGE and immunoblotting using anti–mouse syndecan-1.

**DNA synthesis assay**

PANC-1 cells were cultured in 24-well plates (30,000 cells/well) and allowed to attach for 24 h. After washing with HBSS, cells were switched to serum-free medium containing 0.2% BSA (RIA grade; Sigma-Aldrich) for 48 h. Where indicated, cells were then treated with 8 mU/ml heparinase III and/or PIPLC (in 0.5 mM EDTA–TBS) followed by the addition of fresh heparinase III and/or PIPLC, where used, and incubation continued for 24 h. Monolayers were washed with PBS, fixed with methanol, and lysed with water. DNA was precipitated with 6% (wt/vol) trichloroacetic acid and, following a water wash, was extracted with 0.3 M NaOH (Tanaka et al., 1992). Radioactivity was measured by scintillation counting. By this assay, the EC50 for the PANC-1 response to FG2 was ~0.7 ng/ml.

**MAPK assay**

5 x 10⁵ PANC-1 or MDA-MB-468 cells were plated in 100-mm plates, cultured until confluent, and switched to serum-free medium containing 0.1% BSA for 48 h. In studies with C2C12 cells, cultures were switched to serum-free medium at 70% confluence for 16 h. Where indicated, cells were reseeded to 1 U/ml PIPiC for 1 h. 100 μl/ml of antibody (anti-epidermal growth factor receptor, anti-EGFR, antibody B-B4 (1:200) overnight at 4°C. Membranes were incubated to antibody or control nonimmune antibody (purified mouse IgG) at a final concentration of 4 μg/ml in medium beginning 1 h before exposure to FG2.

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**Antibody blockade of MMP7**

The proteolytic activity of MMP7 was inhibited using mAb 3322 (Chemicon) as described previously (Wroblewski et al., 2003). In brief, cells were exposed to antibody or control nonimmune antibody (purified mouse IgG) at a final concentration of 4 μg/ml in culture medium beginning 1 h before exposure to FG2.

**References**


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