Sustained Down-regulation of the Epidermal Growth Factor Receptor by Decorin

A MECHANISM FOR CONTROLLING TUMOR GROWTH IN VIVO*

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The small leucine-rich proteoglycan decorin interacts with the epidermal growth factor receptor (EGFR) and triggers a signaling cascade that leads to elevation of endogenous p21 and growth suppression. We demonstrate that decorin causes a sustained down-regulation of the EGFR. Upon stable expression of decorin, the EGFR number is reduced by ~40%, without changes in EGFR expression. However, EGFR phosphorylation is nearly completely abolished. Concurrently, decorin attenuates the EGFR-mediated mobilization of intracellular calcium and blocks the growth of tumor xenografts by down-regulating the EGFR kinase in vivo. Thus, decorin acts as an autocrine and paracrine regulator of tumor growth and could be utilized as an effective anticancer agent.

The factors that control tumor progression in vivo involve, among others, the interplay between the invading neoplastic cells and the tumor stroma, a newly formed connective tissue highly enriched in proteoglycans, growth factors, and cytokines. Proteoglycans not only constitute a physical barrier to the invading tumor cells but also influence their behavior by binding and storing growth factors or by activating cell surface receptors (1, 2). Decorin, a prototype member of an expanding family of small leucine-rich proteoglycans, plays key roles in regulating matrix assembly and cell proliferation (3, 4). Most of decorin’s biological interactions occur via the central leucine-rich repeat region, an arch-shaped structure whose concave surface is well suited to bind both globular and nonglobular proteins (5). Our working hypothesis is that the increased expression of decorin around invasive carcinomas represents a mechanism designed to counteract the invading neoplastic cells (6). This hypothesis is based on several observations. For instance, decorin levels are markedly elevated during growth arrest and quiescence, its expression is abrogated by viral transformation, and its transcription is suppressed in most tumorigenic cell lines (4). Upon transgenic expression of decorin, tumor cells with diverse histogenetic backgrounds revert to their normal phenotype; they loose anchorage-indepen- dent growth, fail to generate tumors in immunocompromised animals, and become arrested in G1 (7, 8). Lack of decorin expression is permissive for tumor development insofar as bis- transgenic mice lacking both decorin and the tumor suppressor p53 develop an accelerated lymphoma tumorigenesis (9). We discovered that decorin causes dimerization and autophosphorylation of the epidermal growth factor receptor (EGFR), and that it binds both the soluble EGFR ectodomain and the immunopurified EGFR (10, 11). This interaction triggers a signal cascade leading to activation of mitogen-activated protein kinases (10), mobilization of intracellular calcium (12), up-regulation of p21WAF1CIP1 (p21), a potent inhibitor of cyclin-dependent kinases (13), and ultimately to growth suppression (7, 8, 14).

Despite the above findings, the mechanism by which decorin exerts its cytostatic effects is not understood. It is also unclear how activation of the EGFR by decorin would result in pro- tracted growth suppression. In this investigation, we discovered that decorin leads to a profound and sustained down-regulation of the EGFR. Concurrently, decorin attenuates the EGFR-mediated mobilization of intracellular calcium and blocks the growth of tumor xenografts by acting as an in vivo paracrine growth inhibitor. These results provide an explanation for the long term effects of decorin on tumor suppression and suggest novel therapeutic approaches against cancer.

EXPERIMENTAL PROCEDURES

Materials and Cell Cultures—Media and fetal bovine serum were purchased from Mediatech (Herndon, VA). Hybrid ECL membranes were purchased from Amersham Pharmacia Biotech, and AG1478 was purchased from Calbiochem. Antibodies include polyclonal rabbit antibod- ies against the N-terminal region of decorin (15) and monoclonal antibodies against human p21 (6B6; Pharmingen), against the human EGFR (Ab-12; NeoMarkers, Inc.), and against phosphotyrosine (PY20, Transduction Laboratories). Recombinant human decorin proteoglycan and decorin protein core were purified as before (16).

†‡‡ The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; p21, the cyclin-dependent kinase inhibitor p21WAF1CIP1; [Ca2+]i, cytosolic [Ca2+]i.
Fig. 1. Ectopic expression of decorin causes growth suppression in A431 squamous carcinoma cells via the EGFR. A, survey by Northern blotting of A431 cells stably transfected with full-length human decorin following hybridization with either decorin or GAPDH cDNAs as indicated. Three clones expressing the highest amounts of decorin (AD13, AD14, and AD15 corresponding to lanes 2, 12, and 13, respectively) were studied in detail. B, immunoblotting of media conditioned by either A431 or decorin-expressing clones with an antibody directed against the N-terminal region of decorin. C, immunoblotting of cell lysates from either A431 or decorin-expressing clones as indicated using a monoclonal antibody against human p21.

D, growth curves of A431 and decorin-expressing clones. The number of proliferating cells was determined using a tetramethylbenzidine colorimetric assay with a microplate reader (Promega). Fluorescence-activated cell sorting analysis was performed as described before (8).

Binding Studies with Iodinated EGF—A431, AD13, AD14, and AD15 cells (5 × 10⁴ cells/2 cm²) were plated in complete medium (Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum) on 100-mm dishes (Costar; Corning, NY). EGF binding was conducted with confluent cell cultures essentially as described previously (17). Briefly, cells were washed with ice-cold binding buffer (Dulbecco’s modified Eagle’s medium, 1 mg/ml bovine serum albumin, 25 mM Hepes, pH 7.2), and incubated for 10 min at 4°C. [¹²⁵I]-EGF was added directly to the binding buffer along with the appropriate amount of unlabeled EGF (0.01–50 nM). A large excess (2 μg/ml) of unlabeled EGF was added to replicate wells in the presence of each concentration of [¹²⁵I]-EGF to empirically determine nonspecific binding levels. Cells were incubated for 3 h at 4°C and washed three times with cold binding buffer, and bound [¹²⁵I]-EGF was extracted with 10 mM Tris, pH 7.4, 1 mM EDTA, 0.5% SDS for 15 min at room temperature. [¹²⁵I]-EGF was quantified in a γ-counter (Packard Auto γ 9600). Nonspecific binding, generally <2% of the input radioactivity, could be subtracted from the experimental result. Specific binding was determined by the method of Scatchard using a single site model to determine the K_d and number of EGF receptors per cell. While our data showed a somewhat curvilinear profile indicative of two classes of binding sites, the higher affinity class represented a very small percentage (<3%) of the total and could not be accurately resolved by our analysis. Thus, we applied a single-site linear regression analysis to the data, which yielded better fits with less statistical error.

Co-culture Experiments and Cytosolic [Ca²⁺]i ([Ca²⁺]i) Fluorescence Imaging—A431 cells were cultured on 12-well culture plates (~10⁵ cells/well), while the decorin-expressing clones were cultured in microporous inserts (3 μm) and placed on the top of the AD13 cells. After several days in co-culture, the target cells were tested for growth and EGFR phosphorylation as described above. For fluorescence imaging, the A431 cells and two of their decorin-expressing subclones, AD13 and AD14, were plated onto poly-lysine-coated coverglasses and grown to confluence in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and serum-starved for 24–36 h. The cells were loaded with the Ca²⁺-insensitive hydrophobic acetoxyxymethylester form of furat2 for 25–30 min at room temperature in an extracellular medium containing 121 mM NaCl, 5 mM NaHCO₃, 10 mM Na-HEPES, 4.7 mM KCl, 1.2 mM KH₂PO₄, 2 mM CaCl₂, 10 mM glucose, and 2% bovine serum albumin, pH 7.4, supplemented with 0.003% pluronic acid and sulfipyrazone (200 μM). After loading with furat2AM, cells were washed into an extracellular medium with 0.25% bovine serum albumin supplemented with sulfipyrazone. Subsequently, the coverslips were mounted on the thermostated stage (35°C) of an Olympus IX70 inverted epifluorescence microscope coupled to a high quantum efficiency cooled CCD camera driven by a customized computer program that also controlled a scanning monochromator (DeltaRAM, PTI) to select multiple excitation wavelengths (18). Fura2 fluorescence was measured at 340- and 380-nm excitation using a 400-nm long pass dichroic and a 510/80-nm band pass emission filter. At the end of each experiment, cells were permeabilized with an intracellular medium composed of 120 mM KCl, 10 mM NaCl, 1 mM KH₂PO₄, 20 mM Tris-Hepes, 2 mM MgATP, and 1 μM each of antipain, leupeptin, and pepstatin, pH 7.2, supplemented with 40 μg/ml digitonin, and the fluorescence remaining in the cells was accounted as background fluorescence. Images of the background fluorescence were subtracted from the images collected from intact cells prior to calculation of the fluorescence ratios. To calculate [Ca²⁺]i in nM, [Ca²⁺]i was calibrated in intracellular medium.

In Vivo Studies—To test the ability of decorin to function as a tumor repressor in vivo, decorin-producing cells were co-injected with wild-type A431 human squamous carcinoma cells into 4–6-week-old male nu/nu mice (Taconic) and treated in accordance with institutional guidelines. AD13 cells (A431 cells stably transfected with the human decorin transgene) were mixed with A431 cells in 1:2, 1:4, or 1:8 ratios and injected either alone or in the various combinations described in the text (see Fig. 7 legend). Mice were carefully examined every two or three days for up to 35 days postinjection, and any tumor growth was measured with a microlapicer using the formula V = αb²/2, where a is the width at the widest point and b is the width perpendicular to a. Tumors were frozen in liquid nitrogen, pulverized in a mortar, and boiled in SDS-polyacrylamide gel electrophoresis buffer containing 100 mM β-mercaptoethanol before processing for SDS-PAGE and Western immunoblotting. Parallel samples were analyzed by conventional light microscopy (9).

RESULTS

De Novo Decorin Expression Causes Growth Suppression and Sustained Down-regulation of the EGFR—Transmission of extracellular signals usually starts with binding of a growth factor to surface receptors that in many cases carry an intrinsic tyrosine kinase activity (19, 20). For example, EGF stimulates the formation of homo- and heterodimers with other members of the family of receptor tyrosine kinase, a process that generally leads to growth stimulation (21). However, the proliferation of tumor cells bearing high levels of EGFR, such as A431 squamous (22) or MDA468 mammary (23) carcinoma cells, is stimulated by picomolar but paradoxically suppressed by nanomolar concentrations of EGF, presumably via down-regulation of the EGFR (24). To investigate whether decorin might induce growth suppression by activating a similar pathway, we estab-
lished a number of A431 clones expressing the full-length human decorin driven by the potent cytomegalovirus promoter. All of the decorin-expressing clones (Fig. 1, A and B) exhibited increased levels of endogenous p21 (Fig. 1C) and became growth-retarded (Fig. 1D), with a proportional increase in cells arrested in G1 (Fig. 1E). The addition of either recombinant decorin or decorin protein core (1 μM) caused growth inhibition in wild-type A431 cells, an effect mediated by the EGFR insofar as AG1478 (1 μM), a tyrphostin that specifically inhibits the EGFR kinase (25), abrogated the cytostatic effects induced by either decorin or EGF (not shown).

Three decorin-expressing clones (AD13, AD14, and AD15) were studied in depth. While the steady state levels of EGFR declined by ~40% (42 ± 8%, n = 5), the degree of EGFR activation, as measured by its phosphorylation status, declined by ~95% (Fig. 2, A and B). When the blots were exposed for a short time (2 s), there was no detectable EGFR phosphorylation in the decorin-expressing clones. Only when the blots were saturated (after a 6-s exposure) could tyrosyl phosphorylation of the EGFR be detected (Fig. 2A). The involvement of a non-specific phosphatase was ruled out by experiments in which even relatively high concentrations of Na3VO4 (100 μM) caused no significant change in the state of EGFR phosphorylation (not shown). Moreover, the steady state levels of EGFR mRNA were not significantly altered in the decorin-expressing clones (Fig. 2C), although there was clonal variability in EGFR expression.

When 125I-EGF binding was conducted, the number of binding sites/cell was reduced in all of the decorin-expressing clones to levels comparable with those obtained with immunoblotting (Fig. 2D). Specifically, the binding sites/cell were 2.4 × 10^6 in A431 cells, in contrast to the AD13, AD14, and AD15, which expressed 1.5, 1.1, and 1.2 × 10^6 binding sites/cell, respectively. Interestingly, the affinity constants determined by Scatchard analysis were not significantly different among the various cell lines (Kd = 15–21 nM). These experiments were repeated five times with comparable results.

To further prove that the affinity of the EGFR for EGF was not significantly affected by decorin, quiescent cells were exposed for 10 min to various concentrations of EGF. In agreement with the data presented above, the decorin-expressing cells showed reduced levels of EGFR protein (Fig. 2E, upper
domains to the activated EGFR and becomes tyrosine-phosphorylation of tumor cell growth and EGFR kinase activity.

Thus, decorin induces a substantial reduction in the number of EGFRs without affecting EGFR mRNA steady-state levels. However, decorin causes an even greater suppression of EGFR phosphorylation.

Decorin Does Not Affect the Rate of EGFR Dephosphorylation following EGF Challenge—A potential mechanism for the down-regulation of EGFR includes an increased rate of intracellular dephosphorylation after ligand activation (19, 24, 26).

To address this issue, we exposed quiescent (incubated in serum-free medium for 24 h) A431 or AD13 cells to EGF (40 ng/ml) for 5 min and then chased the cells for 1–40 min in the same medium supplemented with or without AG1478 (2 μM) to block EGFR kinase. While the control samples, treated with MeSO vector alone, showed no appreciable EGFR dephosphorylation within the 40-min chase, the AG1478-treated samples showed a very rapid disappearance of EGFR tyrosyl phosphorylation (Fig. 3A).

Although quantitatively different, the half-life of EGFR tyrosyl phosphorylation was essentially the same in both A431 and AD13 cells, τ1/2 ~ 40 ± 10 s (Fig. 3B). Notably, the EGFR-induced tyrosyl phosphorylation of c-Cbl and the 85-kDa subunit of phosphatidylinositol 3-kinase, both known to be substrates for EGFR kinase, decayed even faster than EGFR (Fig. 3A). Because in these experiments the medium was not changed before adding EGF or AG1478, we conclude that the accumulated decorin synthesized in 24–25 h does not significantly affect the rate of EGFR dephosphorylation following ligand-induced phosphorylation of the EGFR.

Decorin Acts as a Paracrine Suppressor of EGFR Kinase Activity—A potential problem in interpreting data from stably transfected cells is the plausibility of integration of the transgene into a locus that may inadvertently activate or suppress a gene involved in the EGFR signaling pathway. To address this issue, we established co-culture experiments in which either A431 or the decorin-secreting clones (AD13, AD14, or AD15) were cultured in the top chamber of a microporous (3-μm diameter) well, while the bottom wells contained A431 cells. Thus, any soluble molecules, but not cells, would be able to diffuse through the membrane and affect the behavior of the target A431 cells. After 5 days in culture in complete medium containing 10% serum, there was a significant inhibition of A431 cell growth only when the cells were co-cultured in the presence of the decorin-expressing clones at various ratios (Fig. 4, A and B). Concurrent with these changes, there was a marked suppression of EGFR tyrosyl phosphorylation (Fig. 4, C and D).

In a time course experiment, decorin-mediated EGFR phosphorylation peaked at ~2 h and declined to very low levels at 6–8 h (not shown).

Collectively, these results indicate that secreted decorin can induce the same biochemical changes as the endogenous gene and demonstrate a role for decorin as a paracrine regulator of tumor cell growth and EGFR kinase activity.

Cytosolic [Ca2+]c Signaling Induced by EGF Is Attenuated in the Decorin-expressing Cells—One of the downstream effectors of the EGFR is phospholipase Cγ that binds through its SH2 domains to the activated EGFR and becomes tyrosine-phosphorylated. Phospholipase Cγ catalyzes the formation of inositol 1,4,5-triphosphate that in turn mobilizes Ca2+ from internal stores, leading to elevations of [Ca2+]c, a signal that plays fundamental roles in the control of cellular growth and differentiation (27, 28). It has also been shown that down-regulation of EGFR results in attenuated EGFR-activated [Ca2+]c, signals (12, 29).

To test if the EGFR-linked [Ca2+]c signaling could be involved in the changes in expression and phosphorylation of the EGFR described above, we monitored [Ca2+]c in intact, individual cells loaded with the ratiometric fluorescent Ca2+ indicator fura2 (18). The resting [Ca2+]c, did not vary between the wild-type and decorin-expressing cells (48 ± 11 nM in A431, 44 ± 5 nM in AD13, and 49 ± 5 nM in AD14 cells, respectively). The addition of a maximal dose of EGF (100 ng/ml) to A431 cells evoked a large [Ca2+]c increase essentially in every cell (green-red shift, images i–iii in Fig. 5A), and the [Ca2+]c signal displayed a rapid upstroke followed by a slow decay (Fig. 5A, panel iv). In contrast, the decorin-expressing clones showed either no elevation or a slow and small elevation of [Ca2+]c (images i–iii in Fig. 5, B and C). Time course traces of the mean [Ca2+]c changes in AD13 and AD14 cells also showed the attenuated [Ca2+]c signal in response to maximal EGF (Fig. 5, A–C, panels iv).

To further investigate the mechanisms underlying the im-
paired \([\text{Ca}^{2+}]\), signaling in the decorin-expressing cells, we monitored the \([\text{Ca}^{2+}]\) responses evoked by submaximal and maximal doses of EGF and by ATP that elicits \(\text{Ca}^{2+}\) mobilization without activation of EGFR or phospholipase C. A suboptimal dose of EGF (10 ng/ml) elicited \([\text{Ca}^{2+}]\) elevations that were 70\(\pm\)6\(\%\) smaller in AD13 and AD14 cells, respectively, than A431 cells (\(n = 6, p < 0.05\) for both, Fig. 6). The extent of suppression of the elevations evoked by maximal EGF (100 ng/ml) was 50\(\pm\)6\(\%\) in AD13 and AD14 cells, respectively (\(n = 4, p < 0.02\) and <0.01, respectively). In contrast, ATP (200 \(\mu\)M) evoked \([\text{Ca}^{2+}]\) increases that were not significantly different in the wild-type and decorin-expressing clones (AD13, 87\(\pm\)18\(\%\); AD14, 129\(\pm\)19\(\%\) of A431; \(n = 10\)).

These data show impairment of the EGF-activated calcium signaling pathway in the decorin-expressing cells. Because no attenuation of the ATP-induced calcium signals was found in the decorin-expressing cells, changes in the EGFR or a factor proximal to inositol 1,4,5-triphosphate formation should account for the abnormal EGF-activated calcium signaling.

In Vivo Abrogation of Tumor Growth by Decorin—To test whether decorin could act as an autocrine and paracrine inhibitor of in vivo tumor growth, A431 or AD13 cells were injected into nude mice either alone or in various combinations. Notably, the decorin-expressing AD13 cells did not form any tumors (Fig. 7A) even after 3 months of observation following the end point of the experiment. In contrast, the A431 cells and the 1:2 ratio generated tumors essentially with similar kinetics. However, the 1:4 and 1:8 tumors were significantly smaller than controls after 4 weeks of xenograft growth. In a second set of experiments (Fig. 7B), we utilized ratios in which the number of co-injected tumor cells was kept proportional to the wild-type cells. The A431-injected mice (0.25 and 1.0 \(\times\) 10\(^6\) cells) exhibited identical rates of tumor growth and are shown as a single line (Fig. 7B). At no time did the 1:4 (0.25; 1.0 \(\times\) 10\(^6\) A431/AD13 cells) mixtures or the AD13-injected mice show any tumor growth. These animals were allowed to live for an additional 3 months with no signs of tumor.

All of the A431-generated tumor xenografts revealed extensive invasion of the deep fascia and subcutaneous skeletal muscles (Fig. 7C, i), together with copious neovascularization (Fig. 7C, ii). In contrast, the 1:4 ratio showed very sharp margins and no infiltration of the deeper soft tissues (Fig. 7C, iii). In addition, the 1:8 ratio revealed evidence of cytodifferentiation characterized by the formation of tumor nests surrounded by a dense collagenous matrix (Fig. 7C, iv), polarized epithelial cells (Fig. 7C, v), and extensive keratin formation (Fig. 7C, vi). Western immunoblotting of pooled tumor cell lysates from three xenografts revealed that at similar protein concentration (Fig. 7D) there was a significant down-regulation of EGFR phosphorylation in the 1:4 and 1:8 xenografts (Fig. 7E). However, no human decorin was detected in the tumor xenografts.
using sensitive immunoblotting assays and purified decorin as positive controls (not shown). We estimated that the decorin levels in the 1:4 and 1:8 tumor xenografts were \( \leq 10 \) ng/50 mg total cell protein. This is probably due to the poor survival of the decorin-expressing cells, since the AD13 alone never generated tumors in several independent experiments using different tumor cell inocula. Thus, the initial exposure to decorin might have been sufficient to down-regulate the EGFR for a protracted time in the A431 cells.

These data substantiate the co-culture experiments described above and further demonstrate that tumor cells genetically engineered \textit{ex vivo} to express decorin can suppress the growth of EGFR-overexpressing tumor cells in both a paracrine and autocrine fashion.

**DISCUSSION**

Overexpression of ErbB receptor tyrosine kinase correlates with poor prognosis in a wide variety of human cancers (20). We find that exposure of A431 carcinoma cells to a soluble leucine-rich proteoglycan causes a sustained down-regulation of the EGFR. The fact that decorin has no homology with EGF is not surprising, since EGF is promiscuous and encompasses integration of stimuli as diverse as ultraviolet irradiation (30), G protein-coupled serpentine receptors (31), voltage-sensitive calcium channels (32), growth hormone (33), and interleukin-6 (34). On this basis, the EGFR can be considered as a prototype switch point for multiple environmental and internal stimuli (24). Our findings extend these observations and identify decorin as a powerful and long acting biological substance capable of controlling tumor growth by desensitizing the EGFR. Whereas the amount of EGFR and the number of EGF-binding sites decline by 40–50\%, the degree of EGFR tyrosyl phosphorylation is nearly totally abrogated, and the mobilization of intracellular calcium stores, a key EGFR-mediated signaling pathway, is markedly attenuated by decorin. Interestingly, the intracellular dephosphorylation rate of EGFR is not appreciably changed by decorin. Tumor xenografts formed by co-injection of A431 cells and their decorin-expressing counterparts grow more slowly or not at all. This is probably due to a paracrine action of decorin, a mechanism corroborated by coculture experiments. The action of decorin is reminiscent of herceptin, a humanized monoclonal antibody directed against the extracellular domain of ErbB2 (35). Herceptin treatment of ErbB2-overexpressing mammary tumor cells causes suppression of ErbB2 and a concurrent induction of the cyclin-dependent kinase inhibitor p27\(^{kip1}\) and the retinoblastoma-related protein p130, both of which prevent the cells from traversing the S phase (35). Using ErbB2-overexpressing mammary tumor cells, we have recently discovered that decorin also causes suppression of ErbB2 phosphorylation and growth by inducing p21 and cytodifferentiation (44). Thus, decorin appears to act...
as a ligand that suppresses the kinase activity of various ErbB members and ultimately leads to cytostasis.

**Extracellular Matrix Proteins Interact with Receptor Tyrosine Kinase**—The unexpected realization that extracellular matrix molecules can directly serve as ligands for receptor tyrosine kinases has changed the prevailing views about the mechanisms by which cells perceive and respond to extracellular signals (4). The discovery that discoidin domain receptors 1 and 2, two orphan receptor tyrosine kinases, are the receptors for fibrillar collagen opens new perspectives in understanding how matrix molecules affect cell behavior (36, 37). Activation of the discoidin domain receptor kinase requires the native triple helix of collagen, and this interaction differs from typical growth factor/receptor signaling insofar as the kinetics of activation are much slower and protracted in time, similar to the decorin/EGFR interplay. Collagen-induced activation of discoidin domain receptor 2 causes enhanced collagenase (MMP-1) expression, thereby leading to a negative feedback loop that would control the extracellular levels of collagen (36). Because decorin is intimately associated with fibrillar collagen, this interaction differs from typical growth factor/receptor signaling insofar as the kinetics of activation are much slower and protracted in time, similar to the decorin/EGFR interplay. Collagen-induced activation of discoidin domain receptor 2 causes enhanced collagenase (MMP-1) expression, thereby leading to a negative feedback loop that would control the extracellular levels of collagen (36). Because decorin is intimately associated with fibrillar collagen, a complex scenario where multimeric interactions might take place should be contemplated. Specificity would occur at the cellular level, since the expression of EGFR and discoidin domain receptors are quite distinct. An increase in decorin content in the newly formed tumor stroma could trigger a functional interaction with the EGFR, known to be highly expressed in many tumor cells, thereby initiating a signaling cascade that would directly block the cell cycle.

**Mechanisms of EGFR Inhibition by Decorin**—Apart from differential recruitment of tyrosine phosphatases or the negative c-Kit, endocytosis of ligand-receptor complexes is a major mechanism for the gradual attenuation of growth factor signaling (38, 39). Endocytosis of the EGFR requires activation of its intrinsic tyrosine kinase and autophosphorylation (29), both of which are induced by decorin (10, 11). Decorin may lead to deactivation of the EGFR signaling by various plausible mechanisms. First, it might bring to the vicinity of the EGFR a transmembrane tyrosine phosphatase. We do not favor this mechanism, since high concentrations of the phosphatase inhibitor Na3VO4 did not alter the degree of EGFR phosphorylation. Second, decorin might activate a cell surface protein that would in turn bind the EGFR, thereby preventing homo- or heterodimerization and ultimately suppressing its phosphorylation. Our previous results, however, suggest that decorin might bind directly to EGFR and activate the EGFR kinase 

**FIG. 6.** Comparison of EGF- and ATP-induced [Ca2+]c signals in wild type and decorin-expressing A431 cells. Experiments were carried out as described in Fig. 5A, average time course traces calculated from 4–7 separate measurements are shown (see numbers on the graphs). In each measurement, [Ca2+]c responses given by 30–50 individual cells were averaged. A431, AD13, and AD14 cells were challenged with a submaximal (10 ng/ml, left) or a maximal (100 ng/ml, right) dose of EGF. Subsequently, the cells were challenged with ATP (200 μM). B, bar graphs showing the magnitude of the [Ca2+]c responses evoked by 10 ng/ml EGF (left), 100 ng/ml EGF (middle), and 200 μM ATP (right).
high affinity EGFR dimers occurs even in the absence of the ligand and that attachment of a specific ligand stabilizes the dimer complex that otherwise might dissociate into its monomeric (inactive) elements (26). The shift caused by decorin may generate an imbalance between the constitutively active EGFR kinase of dimers and their counteracting phosphotyrosine phosphatases. This mechanism might be sufficient to explain the decline in EGFR activity.

Genetic Evidence from Drosophila for the Presence of Leucine-rich Proteins Blocking the EGFR—Strong support for a biological function of mammalian decorin as a blocker of EGFR activity derives from the discovery of EGFR antagonists in Drosophila. A major function of Drosophila EGFR is to induce a dorsal fate in the follicle cells that surround the oocyte and secrete eggshell (40, 41). Kekkon1, a transmembrane protein that contains six leucine-rich repeats homologous to decorin, accumulates in the dorsal-anterior follicle cells in an EGFR-dependent manner (42, 43). Overexpression of Kekkon1 blocks the formation of dorsal appendages, thus identifying Kekkon1 as a negative regulator of EGFR signaling (42). The activity of Kekkon1 can be overcome by co-expression of an activated EGFR or downstream effectors, but not by molecules, such as rhomboid, that enhance ligand-induced activation of the EGFR (41). Like decorin, Kekkon1 associates with the EGFR, a function that requires the extracellular leucine-rich domain but not its intracellular domain (42). Although the mechanism of action of Kekkon1 appears to differ from that of decorin, the end result is common, i.e. a block of the EGFR-transducing pathway. Thus, the leucine-rich repeats of Kekkon1 and decorin may represent an evolutionarily preserved mechanism by which cells control signaling via the EGFR. While Kekkon1, being a transmembrane protein, would interact primarily with adjacent EGFRs on the same cells, decorin, being a secreted protein, could diffuse distantly and interact with a number of cells expressing various levels of EGFR. Thus, decorin opens the possibility of novel therapeutic approaches in cancer by delivering a natural inhibitor of the EGFR signaling pathway. This would represent an advantage over conventional gene therapies, which generally affect only the cells that have been successfully transduced. A single decorin-transfected cell could conceivably affect several neighboring cells, rendering decorin-mediated growth suppression effective even in systems with low or unpredictable transfection rates.

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REFERENCES

1. Conrad, H. E. (1998) *Heparin-binding Proteins*, Academic Press, Inc., San Diego
2. Bernfield, M., Gótte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) *Annu. Rev. Biochem.* 68, 729–777
3. Iozzo, R. V. (1998) *Annu. Rev. Biochem.* 67, 609–652
4. Iozzo, R. V. (1999) *J. Biol. Chem.* 274, 18843–18846
5. Weber, I. T., Harrison, R. W., and Iozzo, R. V. (1996) *J. Biol. Chem.* 271, 31767–31770
6. Iozzo, R. V. (1997) *Crit. Rev. Biochem. Mol. Biol.* 32, 141–174
7. Santra, M., Skorski, T., Calabretta, B., Lattime, E. C., and Iozzo, R. V. (1995) *J. Biol. Chem.* 270, 32121–32124
8. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993) *Science* 259, 18843–18846
9. Carpenter, G. (1999) *Curr. Opin. Cell Biol.* 11, 190–196
10. Perrimon, N., and Duffy, J. B. (1999) *Cell* 96, 190–196
11. Perrimon, N., and McMahon, A. P. (1999) *Cell* 97, 13–16
12. Ghiglione, C., Carraway, K. L., III, Amundadottir, L. T., Boswell, R. E., Perrimon, N., and Duffy, J. B. (1999) *Cell* 96, 847–856
13. Musacchio, M., and Perrimon, N. (1996) *Dev. Biol.* 178, 63–76
14. Santra, M., Eichstetter, I., and Iozzo, R. V. (August 14, 2000) *J. Biol. Chem.* 10.1074/jbc.M006821200

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10, 251–337
22. Fan, Z., Lu, Y., Wu, X., DeBlasio, A., Koff, A., and Mendelsohn, J. (1995) *J. Cell Biol.* 131, 235–242
23. Filmus, J., Trent, J. M., Pollak, M. N., and Buick, R. N. (1987) *Mol. Cell. Biol.* 7, 251–257
24. Carpenter, G. (1999) *J. Cell Biol.* 146, 697–702
25. Leviitzki, A., and Gazit, A. (1995) *Science* 267, 1783–1788
26. Weiss, F. U., Daub, H., and Ullrich, A. (1997) *Curr. Opin. Genet. Dev.* 7, 80–86
27. Berridge, M., Lipp, P., and Bootman, M. (1999) *Curr. Biol.* 9, R157–R159
28. Clapham, D. E. (1995) *Cell* 80, 259–268
29. Carpenter, G. (1987) *Annu. Rev. Biochem.* 56, 881–914
30. Rossette, C., and Karin, M. (1996) *Science* 274, 1194–1197
31. Daub, H., Weiss, F. U., Wallach, C., and Ullrich, A. (1996) *Nature* 378, 557–560
32. Rosen, L. A., and Greenberg, M. E. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 1113–1118
33. Yamauchi, T., Ueki, K., Tohe, K., Memoto, H., Kine, N., Da, M., Njo, M., Kahashi, M., Kahashi, T., Rai, H., Shima, T., Anuma, Y., Jita, T., Muro, I., Zaki, Y., and Dowaki, T. (1997) *Nature* 390, 91–96
34. Qui, Y., Rav, L., and Kung, H. (1998) *Nature* 393, 83–85
35. Sliwskowski, M. X., Lofgren, J. A., Lewis, G. D., Hotaling, T. E., Fendly, B. M., and Fox, J. A. (1999) *Semin. Oncol.* 26, 60–70
36. Vogel, W., Gish, G. D., Alves, F., and Pawson, T. (1997) *Mol. Cell* 1, 13–23
37. Shrivastava, A., Radziejewski, C., Campbell, E., McGlynn, M., Ryan, T. E., Davis, S., Goldfarb, M. P., Glass, D. J., Lemke, G., and Yanopoulos, G. D. (1997) *Mol. Cell* 1, 25–34
38. Shelly, M., Pinkas-Kramarski, R., Guarino, B. C., Waterman, H., Wang, L.-M., Lyass, L., Alimandi, M., Kuo, A., Lucas, S. S., Pierce, J. H., Andrews, G. C., and Yarden, Y. (1998) *J. Biol. Chem.* 273, 10496–10505
39. Moghal, N., and Sternberg, P. W. (1999) *Curr. Opin. Cell Biol.* 11, 190–196
40. Perrimon, N., and Perkins, L. A. (1997) *Cell* 89, 13–16
41. Perrimon, N., and McMahon, A. P. (1999) *Cell* 97, 13–16
42. Carpenter, G. (1999) *J. Cell Biol.* 146, 697–702
43. Leviitzki, A., and Gazit, A. (1995) *Science* 267, 1783–1788
44. Weiss, F. U., Daub, H., and Ullrich, A. (1997) *Curr. Opin. Genet. Dev.* 7, 80–86
45. Berridge, M., Lipp, P., and Bootman, M. (1999) *Curr. Biol.* 9, R157–R159
46. Clapham, D. E. (1995) *Cell* 80, 259–268
47. Carpenter, G. (1987) *Annu. Rev. Biochem.* 56, 881–914
48. Rossette, C., and Karin, M. (1996) *Science* 274, 1194–1197
49. Daub, H., Weiss, F. U., Wallach, C., and Ullrich, A. (1996) *Nature* 378, 557–560
50. Rosen, L. A., and Greenberg, M. E. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 1113–1118
51. Yamauchi, T., Ueki, K., Tohe, K., Memoto, H., Kine, N., Da, M., Njo, M., Kahashi, M., Kahashi, T., Rai, H., Shima, T., Anuma, Y., Jita, T., Muro, I., Zaki, Y., and Dowaki, T. (1997) *Nature* 390, 91–96
52. Qui, Y., Rav, L., and Kung, H. (1998) *Nature* 393, 83–85
53. Sliwskowski, M. X., Lofgren, J. A., Lewis, G. D., Hotaling, T. E., Fendly, B. M., and Fox, J. A. (1999) *Semin. Oncol.* 26, 60–70
54. Vogel, W., Gish, G. D., Alves, F., and Pawson, T. (1997) *Mol. Cell* 1, 13–23
55. Shrivastava, A., Radziejewski, C., Campbell, E., McGlynn, M., Ryan, T. E., Davis, S., Goldfarb, M. P., Glass, D. J., Lemke, G., and Yanopoulos, G. D. (1997) *Mol. Cell* 1, 25–34
56. Shelly, M., Pinkas-Kramarski, R., Guarino, B. C., Waterman, H., Wang, L.-M., Lyass, L., Alimandi, M., Kuo, A., Lucas, S. S., Pierce, J. H., Andrews, G. C., and Yarden, Y. (1998) *J. Biol. Chem.* 273, 10496–10505
57. Moghal, N., and Sternberg, P. W. (1999) *Curr. Opin. Cell Biol.* 11, 190–196
58. Perrimon, N., and Perkins, L. A. (1997) *Cell* 89, 13–16
59. Perrimon, N., and McMahon, A. P. (1999) *Cell* 97, 13–16
60. Ghiglione, C., Carraway, K. L., III, Amundadottir, L. T., Boswell, R. E., Perrimon, N., and Duffy, J. B. (1999) *Cell* 96, 847–856
61. Musacchio, M., and Perrimon, N. (1996) *Dev. Biol.* 178, 63–76
62. Santra, M., Eichstetter, I., and Iozzo, R. V. (August 14, 2000) *J. Biol. Chem.* 10.1074/jbc.M006821200