Decorin Activates the Epidermal Growth Factor Receptor and Elevates Cytosolic Ca\(^{2+}\) in A431 Carcinoma Cells*

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Several independent lines of evidence have implicated decorin, a small leucine-rich proteoglycan, in the inhibition of cell proliferation. However, the mechanism by which decorin mediates its effect on cell proliferation is unclear. Here we report, for the first time, decorin-mediated increases in intracellular Ca\(^{2+}\) levels of single A431 cells. The effects of decorin persisted in the absence of extracellular Ca\(^{2+}\), but were blocked by AG1478, an epidermal growth factor (EGF)-specific tyrosine kinase inhibitor, and by down-regulation of the EGF receptor. The effects of decorin were not mimicked by the structurally homologous protein, biglycan. Our results indicate a novel action of decorin on the EGF receptor, which results in mobilization of intracellular Ca\(^{2+}\) providing a possible mechanism by which decorin causes growth suppression.

Stimulation of phospholipase C (PLC)\(\textsuperscript{\textsc{i}}\) by cell surface receptors results in the generation of the second messenger 1,4,5-trisphosphate and consequent activation of Ca\(^{2+}\) channels located within the membrane of intracellular Ca\(^{2+}\) stores (1). Growth factors, such as epidermal growth factor (EGF) activate receptor tyrosine kinases, which dimerize, autophosphorylate, and generate inositol 1,4,5-trisphosphate via phosphorylation and consequent stimulation of the PLC-\(\gamma\) isoform (2). The ensuing increases in the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{cyt}}\)), which are often both temporally and spatially complex (3), are implicated in a wide range of cellular processes.

Decorin is a key regulator of extracellular matrix assembly and is a member of an expanding family of proteoglycans characterized by a central domain with tandem leucine-rich repeats (4). Molecular modeling has revealed an arch-shaped molecule with a concave surface capable of interacting with various other proteins (5). Targeted disruption of the decorin gene leads to mice with a skin fragility phenotype and altered collagen fibril morphology (6), thereby providing genetic evidence that decorin is a “modifier” of collagen fibrillogenesis during development.

Increasing evidence suggests an inhibitory role for this secreted proteoglycan in cell proliferation (7). In a variety of cell types, overexpression of decorin inhibits cell growth (8, 9). Furthermore, levels of decorin are markedly increased in quiescent cells and reduced in transformed cells (10, 11). Decorin may also play an important role in tumorigenesis, because not only is decorin expression decreased in various tumor cell types, but secretion of decorin by stromal cells surrounding tumors is increased (12). This may represent a response by the host to reduce the rate of carcinoma spread.

Although decorin overexpression is associated with an up-regulation of p21 (13), an inhibitor of cyclin-dependent kinases required for progression through the cell cycle (14), upstream targets for decorin, and the exact mechanism by which decorin inhibits cellular growth are unknown. Recently, we have shown that decorin stimulates phosphorylation of the EGF receptor and activation of the mitogen-activated protein (MAP) kinase pathway (15). Because EGF receptors can couple to PLC, decorin may also elevate [Ca\(^{2+}\)]\(_{\text{cyt}}\), implicating a role for Ca\(^{2+}\) in the inhibitory effects of decorin on cell proliferation. Here, we report for the first time that activation of the EGF receptor by decorin results in elevation of [Ca\(^{2+}\)]\(_{\text{cyt}}\), in single A431 squamous carcinoma cells. Therefore, Ca\(^{2+}\) signals may be important in mediating decorin-induced growth inhibition, which ultimately may retard tumor progression in vivo.

EXPERIMENTAL PROCEDURES

Materials—Human squamous carcinoma A431 cells were obtained from ATCC (Rockville, MD). Highly purified human recombinant decorin and biglycan synthesized by human fibrosarcoma HT1080 cells using the vaccinia virus/T7 phage expression system were purified as described previously (16, 17). Fura-2 and Pluronic F-127 acid were from Tefabs, ionomycin was from Calbiochem, and human recombinant EGF was from Life Technologies, Inc. All other reagents were from Sigma or Fisher.

Cell Culture—A431 cells were cultured on glass coverslips coated with poly-d-lysine (5 \(\mu\)g/cm\(^2\)) and maintained at 37 °C in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 50 \(\mu\)g/ml streptomycin in an atmosphere of 5% CO\(_2\)/air (5%:95%). At 70% confluence, the cells were cultured for a further 48 h in serum-free medium prior to imaging experiments.

Single Cell Ca\(^{2+}\) Imaging—Imaging experiments were performed at room temperature in a medium composed of 121 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO\(_4\), 1.2 mM KH\(_2\)PO\(_4\), 10 mM glucose, 2 mM CaCl\(_2\), 0.25% (w/v) bovine serum albumin, and 20 mM HEPES (pH 7.4 at room temperature). [Ca\(^{2+}\)]\(_{\text{cyt}}\) was determined by loading the cells with fura-2 by incubation with fura-2/acetoxymethyl ester (5 \(\mu\)M) in the presence of Pluronic F-127 acid (0.02% w/v) for 20 min. Coverslips were then mounted on an inverted epifluorescence microscope, and fura-2 was excited at 340 and 380 nm and emission (420–600 nm) collected using a charged coupled device camera. Autofluorescence was determined following quench of fura-2 by Mn\(^{2+}\) in Ca\(^{2+}\)-free medium supplemented with 1 mM MnCl\(_2\) and 2 \(\mu\)M ionomycin. The ratio of the fluorescence at 340 and 380 nm was converted to [Ca\(^{2+}\)]\(_{\text{cyt}}\) according to Grynkiewicz et al. (18).
RESULTS AND DISCUSSION

Decorin Increases [Ca\(^{2+}\)]\(_i\) in Single A431 Cells—We have previously shown that decorin-mediated growth inhibition in A431 cells is associated with phosphorylation of the EGF receptor (15). Generally, EGF stimulates cell growth and proliferation, but the proliferation of certain cells is paradoxically inhibited by this growth factor (19). The prototype of such cells is the A431 human squamous carcinoma cell, which expresses a high number of EGF receptors (20). Because activation of EGF receptors results in autophosphorylation and can potentially stimulate PLC-\(\gamma\), we examined the effects of decorin on [Ca\(^{2+}\)]\(_i\) in individual fura-2-loaded A431 cells. Fig. 1 demonstrates typical [Ca\(^{2+}\)]\(_i\) increases in response to stimulation of A431 cells with decorin. From five separate cell passages, decorin increased [Ca\(^{2+}\)]\(_i\) in 49 ± 12% of cells. In the 498 responding cells, [Ca\(^{2+}\)]\(_i\) increased from a resting level of 30 ± 1 nM to a peak of 123 ± 3 nM. Similar responses were observed after removal of Ca\(^{2+}\) from the extracellular medium (Fig. 1C). These results indicate that decorin mobilizes Ca\(^{2+}\) primarily from intracellular stores as opposed to directly stimulating Ca\(^{2+}\) entry across the plasma membrane.

Biglycan is another member of the family of small leucine-rich proteoglycans sharing 57% amino acid homology with decorin (4). In contrast to decorin, biglycan did not cause growth inhibition (9) and did not affect [Ca\(^{2+}\)]\(_i\) (Fig. 2). Taken together, these data support a specific effect of decorin on A431 cells.

Decorin-induced Responses are Prevented by AG1478, a Specific Inhibitor of EGF Receptor Tyrosine Kinase—To establish
whether decorin-mediated increases in $[\text{Ca}^{2+}]$$_i$ were via activation of the EGF receptor, we examined the effects of the tyrphostin AG1478, an EGF-selective tyrosine kinase inhibitor on responses to EGF (21) and decorin (15). From nine separate cell passages, EGF elevated $[\text{Ca}^{2+}]$$_i$ in 90.64% of cells from a resting level of 28 ± 1 nM to a peak level of 191 ± 63 nM (1369 responding cells out of 1492 cells, Fig. 3A). Pretreatment of the cells with AG1478 (2 μM) reduced the number of cells responding to EGF (Fig. 3B) by 97 ± 2% (27 responding cells out of 533 cells from five separate cell passages). AG1478 completely abolished the $[\text{Ca}^{2+}]$$_i$ responses to decorin (0 responding cells out of 499 cells from two separate cell passages; Fig. 3C). Notably, we used a concentration of AG1478 that is about 1/50 of that required to inhibit c-src kinase activity (21) but that effectively blocks activation of the EGF receptor kinase and MAP kinase caused by either EGF or exogenous decorin (9). Moreover, we found that MAP kinase activation by platelet-derived growth factor was unaffected by the same concentrations of AG1478 (9). In addition, AG1478 did not affect $[\text{Ca}^{2+}]$$_i$ responses to ATP. In five separate cell passages, ATP (200 μM) elevated $[\text{Ca}^{2+}]$$_i$ by 776 ± 44 nM (907 cells) and 870 ± 14 nM (670 cells) in the absence and the presence of AG1478, respectively. The inhibitory effects of AG1478 on EGF responses were reversed if AG1478 was washed out 45 min prior to stimulation (not shown).

Decorin Responses Are Inhibited by EGF Receptor Down-regulation—We next examined the effects of EGF and decorin on $[\text{Ca}^{2+}]$$_i$ in A431 cells in which EGF receptors had been down-regulated. Prolonged exposure of cells to EGF has been shown to reduce the number of EGF receptors at the cell surface (22). Under these conditions, the number of cells responding to EGF (Fig. 4A) and decorin (Fig. 4B) was reduced by 96 ± 3% (4 responding cells out of 255 cells from three separate cell passages) and 99% (3 responding cells out of 424 cells from two separate cell passages), respectively. Down-regulation of EGF receptors reduced $[\text{Ca}^{2+}]$$_i$ increases in response to ATP by 58 ± 9%.

Our results show that decorin mediates $[\text{Ca}^{2+}]$$_i$ increases in A431 cells. These responses may occur in vivo, because the concentration of decorin used in the present study (~1 μM) is much lower than that estimated to be bound to dermal collagen, the site of interaction between keratinocytes and the mesenchyme. The effects of decorin on $[\text{Ca}^{2+}]$$_i$ are unlikely to be mediated by activation of integrins, which has been reported previously for other extracellular matrix constituents, because decorin does not contain the tripeptide RGD consensus sequence characteristic of integrin-binding proteins (23). Recent studies have demonstrated that syndecan-4, a transmembrane proteoglycan, can activate protein kinase C (24, 25). Our results, however, are consistent with decorin activating PLC-γ via the EGF receptor, because decorin mediates $[\text{Ca}^{2+}]$$_i$ increases that are abolished by either inhibition of EGF receptors with AG1478 or down-regulation of EGF receptors. Because
EGF also inhibits proliferation in this cell type, our results provide a possible mechanism by which decorin mediates its negative effects involving direct activation of the EGF receptor and increases in cytosolic Ca$^{2+}$.

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