Decorin Is a Biological Ligand for the Epidermal Growth Factor Receptor*

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Renato V. Iozzo‡§§, David K. Moscatello§, David J. McQuillan†, and Inge Eichstetter‡

From the ‡Department of Pathology, Anatomy, and Cell Biology and §Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 and †Center for Extracellular Matrix Biology, Institute of Biosciences and Technology, Texas A&M University, Houston, Texas 77030

Communications

Ectopic expression of decorin induces profound cyto-static effects in transformed cells with diverse histogene-tic backgrounds. The mechanism of action has only recently begun to be elucidated. Exogenous decorin activates the epidermal growth factor (EGF) receptor, thereby triggering a signaling cascade that leads to phosphorylation of mitogen-activated protein (MAP) kinase, induction of p21, and growth suppression. In this study we demonstrate a direct interaction of decorin with the EGF receptor. Binding of decorin induces dimerization of the EGF receptor and rapid and sustained phosphorylation of MAP kinase in squamous carcinoma cells. In a cell-free system, decorin induces autophosphorylation of purified EGF receptor by activating the receptor tyrosine kinase and can also act as a substrate for the EGF receptor kinase itself. Using radio-ligand binding assays we show that both immobilized and soluble decorin bind to the EGF receptor ectodomain or to purified EGF receptor. The binding is mediated by the protein core and has relatively low affinity (Kd ~87 nM). Thus, decorin should be considered as a novel biological ligand for the EGF receptor, an interaction that could regulate cell growth during remodeling and cancer growth.

Decorin plays a pivotal role in regulating the proper assembly of collagenous matrices and in the control of cell proliferation (1). Most of its biological interactions occur via the central leucine-rich repeat region, which is thought to fold into an arch-shaped structure (2). Because of the ability of decorin to bind fibrillar collagen and to delay in vitro fibrillogenesis (3), this proteoglycan is regarded as a key modulator of matrix assembly. Genetic studies utilizing decorin null mice have indeed proved a major role for decorin in the homeostasis of dermal collagen. Although the nullizygous animals grew to adulthood without any overt pathology, a close analysis revealed a skin fragility phenotype; the dermal collagen exhibited aberrant organization of fibrils with abnormal packing and a great variability in diameter (4). Another important role of decorin is linked to its ability to inhibit transforming growth factor-β, which in turns blocks cell proliferation (5).

There is also mounting evidence that decorin is involved directly in the control of cell growth. 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 (6). De novo expression of decorin induces a generalized growth suppression by activating p21, a potent inhibitor of cyclin-dependent kinase activity (7–9). Decorin activates the EGF receptor (EGFR) (10) that in turn triggers a signal cascade leading to activation of MAP kinases, mobilization of intracellular calcium (11), up-regulation of p21, and growth suppression.

In this report we demonstrate a direct interaction between the EGFR and decorin. Exogenous decorin causes dimerization of the EGFR in A431 squamous carcinoma cells. Decorin induces autophosphorylation of the EGFR by activating the EGFR tyrosine kinase in a cell-free system and can also act as a substrate for the EGFR kinase itself. Both immobilized and soluble decorin bind to the soluble EGFR ectodomain or to immunopurified EGFR. The binding is solely mediated by the protein core and has relatively low affinity (Kd ~87 nM). Thus, decorin should be considered as a novel biological ligand for the EGFR.

EXPERIMENTAL PROCEDURES

Materials—Media and fetal bovine serum were obtained from Hyclone Laboratories, Inc. (Logan, UT). [γ-32P]ATP (6000 Ci/mmol) and Hybrid ECL membranes were from Amersham Pharmacia Biotech. The EGFR preparation purified from A431 cells by immunoaffinity chromatography (Sigma) contained ~500 units of active EGFR; 1 unit catalyzes the incorporation of 1 pmol of phosphate/min from [γ-32P]ATP into poly(Glu,Tyr) at 30 °C. Cross-linking Studies with Soluble EGFR Ectodomain—Confluent A431 cells, which were rendered quiescent by serum deprivation for 36 h, were incubated with decorin or decorin protein core (1 µg) or EGF (16 ng) or various combinations at 4 °C for 1 h. The cells were washed extensively and incubated for 10 min with the membrane-impermeable cross-linker bis[sulfosuccinimidyl]suberate (BS3, Pierce). The cell lysates were separated in 3–15% SDS-PAGE and subjected to immunoblotting (10) with anti-EGFR antibody. The anti-phospho-MAP kinase antibody (New England Biolabs) detects p42 and p44 MAP kinases (Erk1/Erk2) only when they are catalytically activated by phosphorylation at Thr286 and Tyr388.

In Vitro Phosphorylation of the EGFR Kinase—Constant amounts (~300 ng) of immunopurified EGFR were preincubated with buffer alone or containing increasing concentrations (5–20 µg) of decorin, its decorin protein core, or collagen type I. After 15 min of incubation in kinase buffer (20 mM HEPES, pH 7.4, 2 mM MnCl2, 0.01% BSA, 15 µM ATP) 1 µCi of [γ-32P]ATP and 0.2% Nonidet P-40 was added to reach a final volume of 60 µl. The mixture was incubated for an additional 10 min, stopped by boiling in SDS buffer, and analyzed by SDS-PAGE. Phosphorylated proteins were visualized by autoradiography. Control samples omitting either [γ-32P]ATP or EGFR showed no activity.

Binding Studies with Soluble EGF Ectodomain—Because A431 cells synthesize a soluble form of EGFR of ~105 kDa lacking the
Transmembrane and intracytoplasmic domains (12), serum-free medium conditioned by confluent A431 cells was concentrated by Centriprep-50 and incubated with decorin, or its protein core immobilized on nitrocellulose filters, washed, and subjected to immunodetection with anti-EGFR antiserum. Briefly, serial dilutions of BSA, decorin, or its protein core were slot-blotted onto ECL nitrocellulose, blocked overnight at 4 °C with 5% fetal bovine serum and 5% nonfat milk, and washed several times in TBS-T (Tris-buffered saline, 25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4). The membranes were incubated with the serum-free medium conditioned by the A431 cells, washed 3 more times, incubated with an antibody raised against the N-terminal sequence LEEKK of the human EGFR, and affinity-purified on a peptide linked to Sepharose. This antibody would not interfere with the binding because it recognizes the N-terminal end of the EGFR. As additional control, we used a mouse monoclonal antibody 225 raised against the EGF-binding domain of the EGFR (13).

Interaction Between Decorin and Purified EGF Receptor—We utilized columns containing nickel-nitriilotriacetic acid (Ni-NTA) as ligand (Qiagen) since the recombinant human decorin contains an N-terminal tag with 6 histidine residues (His6) that allows a rapid and efficient purification via Ni-NTA affinity chromatography (14). First we phosphorylated the immunopurified EGFR to reach a specific activity of ~1.7 × 10⁶ cpm/mol using the in vitro phosphorylation assay described above. Following purification by Sephadex G-50 chromatography, constant amounts of ³²P-labeled EGFR were incubated with increasing concentrations of decorin or its core protein for 30 min at 4 °C under gentle agitation. The spin columns were equilibrated with 3 column volumes of binding buffer (300 mM NaH₂PO₄, pH 8.0, 300 mM NaCl), and then the samples were applied and spun at 750 × g for 5 min. Following two consecutive washes, the bound decorin-EGFR complexes were eluted with buffer containing 250 mM imidazole. Parts of the fractions were counted in a scintillation counter, and parts were analyzed by SDS-PAGE and autoradiography. Radioligand binding assays were performed as described before (15). Decorin was coated in removable Immulon 4HXB wells (Dynex Technologies, Chantilly, VA), and binding of ³²P-labeled EGFR was measured in TBS supplemented with 2 mM CaCl₂, 2 mM MgCl₂, 0.02% NaN₃, and 1 mg/ml heat-inactivated BSA following incubation under gentle shaking (80 rpm) for 4–14 h. Reversible binding was demonstrated by incubation with 100-fold molar excess EGFR. After incubation the wells were washed with ice-cold TBS-T and then counted in toto. Scatchard plots were generated using the Ligand program as described before (16).

RESULTS AND DISCUSSION

Decorin Causes Dimerization of the EGF Receptor—It is well established that the general mechanism transmitting extracellular signals starts with binding of a growth factor to a cell surface receptor that in many cases carries an intrinsically tyrosine kinase activity (17). For example, EGF stimulates the formation of non-covalent homo- and heterodimers with other members of the family of receptor tyrosine kinase (17). The growth of some tumor cells bearing high levels of EGF receptors is paradoxically suppressed. For instance, A431 vulvar squamous carcinoma cells are stimulated to grow with picomolar amounts of EGF but are markedly growth inhibited with nanomolar amounts of the growth factor (18). EGF treatment of A431 cells causes a protracted induction of p21(19, 20), and similarly, antibodies that recognize the EGF-binding region of the EGFR inhibit the proliferation of normal and transformed cells (13, 21–24). Thus, we reasoned that decorin might induce growth suppression by activating a similar signal-transducing pathway. To test whether decorin could cause dimerization of the EGFR, we added purified decorin proteoglycan or its protein core to quiescent A431 cells at 4 °C for 1 h, washed extensively, and incubated for 10 min with BS3, a non-cleavable, membrane-impermeable cross-linker. Decorin induced significant dimerization of the EGFR (Fig. 1A, lanes 4 and 5), estimated to be ~40% of that induced by EGF (Fig. 1A, lane 2). There were no additive effects when decorin or its protein core were added concurrently with EGF (Fig. 1, A, lanes 6 and 10, and B, lane 6). The protein core of decorin was capable of mediating the full effect of the decorin proteoglycan (Fig. 1B).

To investigate this further, we iodinated decorin and performed similar cross-linking experiments followed by immunoprecipitation. We found a very high M₉ complex that could not penetrate the gel (not shown), further suggesting the decorin might be directly involved in binding to the EGFR.

Decorin Causes Rapid and Sustained Activation of MAP Kinase and Induces Autophosphorylation of Purified EGF Receptor—To investigate whether decorin induces rapid and sustained activation of the MAP kinase signal-transducing pathway, we exposed quiescent A431 cells to 1 μM decorin or its protein core for either 18 h or 10 min. As positive and negative controls we used EGF (16 nM) and collagen (1 μM), respectively. The results showed both a sustained (Fig. 2A) and rapid (Fig. 2, B and C) activation of the MAP kinase signal pathway, which leads to prolonged activation of the endogenous p21 and block of the cells in G₁ (10). To more directly investigate the interaction between the EGFR and decorin, we used EGFR purified by immunoaffinity chromatography from plasma membranes of A431 cells (25). The EGFR was not exposed to EGF during the purification procedure, and thus it contains optimal kinase activity and is suitable for both binding and in vitro kinase assays. First, we established by Western immunoblotting the purity of the EGFR and the lack of degradation products. A single ~170-kDa band was seen using an antibody against the N terminus of the EGFR (Fig. 2D). When the EGFR was subjected to in vitro kinase assay there was a dose-dependent induction of EGFR autophosphorylation only in the presence of decorin (Fig. 2E, lanes 2–4) or its protein core (Fig. 2E, lanes 5–7) but none in the presence of collagen (Fig. 2E, lanes 8–10). Of note, the EGFR kinase efficiently phosphorylated decorin protein core as seen by the appearance of the two ~42- and 46-kDa proteins seen only when the protein core was added (Fig. 2E, lanes 5–7). No significant phosphorylation of the decorin proteoglycan (which would have appeared as a ~100-kDa band) was noted, indicating that the actual phosphorylation of the protein core may be inhibited by the dermatan sulfate chain. The binding of decorin to the EGFR was totally abolished by thermal denaturation (80 °C for 15 min) of both decorin and its protein core (not shown). The same treatment of the EGFR abolished the binding to decorin. Thus, proper protein folding is required for this interaction.

Decorin Interacts with the Soluble Ectodomain of the EGF Receptor—The rationale for these studies is based on the observation that A431 cells synthesize and release into the medium a soluble form of EGFR of ~105 kDa lacking the transmembrane and intracytoplasmic domains (12, 26). This soluble protein is derived from the 2.8-kilobase mRNA transcribed...
Decorin activates MAP kinases in A431 cells and induces autophosphorylation of purified EGF receptor. A–C, Western immunoblotting of total cell lysate probed with an antibody against the phosphorylated forms of Erk1/Erk2 MAP kinases (MAPK) using a polyclonal anti-phospho-p44/42 MAP kinase antibody (New England BioLabs). Cells were rendered quiescent by serum starvation for 2 days and then treated with 1 μg decorin, protein core, or collagen type I as indicated. EGF (16 nM) was used as a positive control (10). D, Western immunoblot of immunopurified EGFR using an antiseraum against the N-terminal domain of the EGFR. E, in vitro phosphorylation assay using immunopurified EGFR and decorin, its protein core, or collagen. Constant amounts (~300 ng) of immunopurified EGFR were preincubated with buffer alone (lane 1) or containing decorin (lanes 2–4; at 5, 10, and 20 μg, respectively), decorin protein core (lanes 5–7; at 5, 10, and 20 μg, respectively), or collagen (lanes 8–10; at 5, 10, and 20 μg, respectively). After 15 min in kinase buffer, 1 μCi of [γ-32P]ATP and 0.2% Nonidet P-40 was added. The mixture was incubated for an additional 10 min, stopped by boiling in SDS buffer, and analyzed by SDS-PAGE and autoradiography.

from a rearranged EGFR gene on chromosome 7 (27). As the extracellular domain has both a high Cys content and large amount of oligosaccharides, the ectodomain is quite stable and highly resistant to proteolysis. It binds with high affinity EGF (12, 26), and available stoichiometry data show that 1 mol of EGF binds 1 mol of EGFR. First, we tested the purity of the medium conditioned for 48 h by A431 cells by performing Western immunoblotting. A single band of ~105 kDa was detected using the anti-EGFR antisera (Fig. 3A). This material was used in slot-blot overlay assays in which both proteins (the immobilized and the soluble ligand) are under native conditions. Serum-free medium conditioned 48 h by A431 cells was concentrated 8-fold by Centricon-50 and incubated with decorin, its protein core, or BSA immobilized on nitrocellulose filters, washed, and subjected to immunodetection with anti-EGFR antisera (28). Both protein core (Fig. 3B) and decorin (Fig. 3C) bound specifically to soluble EGFR ectodomain, in contrast to BSA, which was unreactive. Of note, a chimeric biglycan/decorin protein containing the N terminus of biglycan and the remaining decorin gave identical results (not shown).

Thus, the binding of EGF is not mediated by the N terminus since biglycan did not cause activation of EGFR (10). When similar slot-blot overlay assays were tested with monoclonal antibody 225 that reacts specifically with the EGF binding region of the EGFR (13), there was no reactivity (not shown), suggesting that decorin might bind near or perhaps in the same location where EGF binds.

To further corroborate the above results, we immobilized on nitrocellulose scalar amounts of media conditioned for various periods of time by A431 cells and performed overlay assays with soluble decorin followed by Western immunoblotting (29). The results showed specific binding of decorin to serum-free medium conditioned by A431 cells, which contains the EGFR ectodomain (Fig. 3D). As negative and positive controls we used either Dulbecco’s modified Eagle’s medium (Fig. 3D) or scalar dilutions (~50 ng) of purified decorin (Fig. 3E), respectively. Our results show that decorin interacts specifically with the ectodomain of the EGFR in either solution or immobilized on nitrocellulose membranes.

Decorin Interacts with Purified EGF Receptor—Next we sought to investigate whether soluble decorin could interact with the EGFR under physiological salt concentrations and in solution. The rationale for this approach is that decorin contains an N-terminal His tag that allows a rapid and efficient purification via Ni-NTA affinity chromatography (14). Constant amounts of 32P-labeled EGFR were incubated with increasing concentrations of decorin or its core protein for 30 min at 4 °C under gentle agitation. The Ni-NTA columns were equilibrated with 3 column volumes of binding buffer, and then the samples were applied and spun at 750 × g for 5 min. Following two consecutive washes, the bound decorin-EGFR complexes were eluted with buffer containing 500 mM imidazole. In two independent experiments there was specific binding of the EGFR to decorin, since the amount of radioactive EGFR increased proportionally to the amount of interacting (Ni-NTA-bound) decorin (Fig. 4, A–C). Quantitation of both experiments gave a strong correlation with r² = 0.961 and p < 0.0005 (Fig. 4D).

Finally, to determine more precisely the binding affinity we utilized a radioligand binding assay where increasing concentrations of 32P-labeled EGFR were tested on Immulon wells.
Ni-NTA column via the polyhistidine tag. Specifically with decorin, which in turn binds with high affinity to the concentrations of decorin protein core. The radiolabeled EGFR interacts in such experiments is chard plots (not shown). Notably, the affinity constant obtained shown). In parallel experiments where 10-fold more decorin decorin is either immobilized on nitrocellulose membranes or coated with decorin protein core (22 pmol). Binding of EGFR to decorin protein core. The values represent the average of two independent experiments showing the amount of mean values of two independent experiments. Linear regression, correlation coefficient \( r^2 \), and \( p \) values were obtained using the Regression Wizard Program of the Sigma Plot 4 Package (Jandel). Saturation curve \( E \) and Scatchard plot \( F \) for the binding or \( ^{32}\text{P} \)-labeled EGFR to decorin protein core are shown. The EGFR was tested at 0.1–3.2 pmol \((1.7 \times 10^{17} \text{cpm} / \text{mol})\) on removable wells coated with 1 \( \mu \text{g} \) (−22 pmol) of decorin protein core. The values represent the average of two independent experiments. The cumulative data from five experiments gave a \( K_d \) \( \sim 87 \text{nM} \pm 7.5 \). coated with decorin protein core (22 pmol). Binding of EGFR to decorin protein core was saturable (Fig. 4E), and a Scatchard plot (Fig. 4F) gave a single straight line with \( K_d \) \( \sim 87 \pm 7.5 \text{nM} \) \((n = 5)\). The binding of decorin to the radiolabeled EGFR was totally abolished by 100-fold molar excess of cold EGFR (not shown). In parallel experiments where 10-fold more decorin protein core was used to coat the wells, the binding of EGFR was rapidly saturable, and the data also yielded linear Scatchard plots (not shown). Notably, the affinity constant obtained in such experiments is \( 90\)-fold lower than that reported for the low affinity binding sites for EGF \((\sim 1 \text{nM})\) and \( 900\)-fold lower than the high affinity receptor for EGF \((\sim 0.1 \text{nM})\) (27). 

Conclusions—We have demonstrated that decorin protein core causes a rapid phosphorylation of the EGFR in A431 cells, which leads to a specific activation of the MAP kinase signal pathway and to induction of endogenous p21. Several lines of evidence support a specific protein/protein interaction between decorin and the EGFR. We demonstrate for the first time that decorin is capable of inducing dimerization of the EGFR in live cells, a physiologic phenomenon previously shown to be a prerequisite to receptor activation. The specific binding occurs when decorin is either immobilized on nitrocellulose membranes or free in physiologic salt solutions. In a cell-free system, decorin induces autophosphorylation of purified EGFR by activating the receptor tyrosine kinase and can also act as a substrate for the EGFR kinase itself, although it is unlikely to be a substrate \textit{in vivo} since decorin is an extracellular molecule. Decorin is capable of inducing EGFR tyrosine kinase and that both the binding and activation require properly folded protein moiety. Notably, DDR1 and DDR2, two “orphan” receptor tyrosine kinases, have been shown to be the receptors for fibrillar collagen (28, 30). Similarly to the decorin/EGFR interaction, stimulation of the DDR receptor tyrosine kinase activity requires the native triple helical structure of collagen and occurs over an extended period of time (30). The unexpected realization that extracellular matrix molecules can directly serve as ligands for receptor tyrosine kinases may change prevailing views about the mechanisms by which cells perceive and respond to the extracellular matrix signals. Because decorin is intimately associated with fibrillar collagen, a complex scenario where multimeric interactions take place in an integrin-independent manner should be considered. An increase in decorin content in the newly formed tumor stroma could potentially trigger a functional interaction with the EGFR, known to be highly expressed in most tumor cells, which would in turn start a signaling process that directly influences the cell cycle machinery.

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