Decorin Evokes Protracted Internalization and Degradation of the Epidermal Growth Factor Receptor via Caveolar Endocytosis*

Jing-Xu Zhu¹, Silvia Goldoni¹, Gregory Bix², Rick T. Owens³, David J. McQuillan¹, Charles C. Reed⁴, and Renato V. Iozzo⁴*²

From the *Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania 19107, the †LifeCell Corporation, Branchburg, New Jersey 08876, and the The Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Decorin inhibits the epidermal growth factor receptor (EGFR) by down-regulating its tyrosine kinase activity, thereby blocking the growth of a variety of transformed cells and tumor xenografts. In this study we provide evidence that decorin directly binds to the EGFR causing its dimerization, internalization, and ultimately its degradation. Using various pharmacological agents to disrupt clathrin-dependent and -independent endocytosis, we demonstrate that decorin evokes a protracted internalization of the EGFR primarily via caveolar-mediated endocytosis. In contrast to EGF, decorin targets the EGFR to caveolae, but not to early or recycling endosomes. Ultimately, however, both EGF- and decorin-induced pathways converge into late endosomes/lysosomes for final degradation. Thus, we have discovered a novel biological mechanism for decorin that could explain its anti-proliferative and anti-oncogenic mode of action.

Decorin, the prototypic member of an expanding family of small leucine-rich proteoglycans (1), is implicated in modulating collagen fibrillogenesis (2, 3) and cell growth and survival (4–8). The mechanism of decorin action has begun to be elucidated by the discovery that decorin interferes with epidermal growth factor receptor (EGFR) signaling (9, 10). Decorin leads to a protracted down-regulation of EGFR tyrosine kinase (11) and other members of the ErbB family of receptor tyrosine kinase (12), and causes an attenuation of the EGFR-mediated mobilization of intracellular calcium (11). Decorin induces expression of the endogenous cyclin-dependent kinase inhibitor p21WAF1/CIP1 (13, 14) and a subsequent arrest of the cell in the G1 phase of the cell cycle (15). These cytostatic effects occur in a wide variety of tumor cell lines (5, 14) and can also affect murine tumor cells (5) and normal human cells, such as endothelial cells (16) and macrophages (17). During quiescence, decorin expression is markedly up-regulated in most normal diploid cells, whereas its expression is nearly abolished in most transformed cells (2, 18–20). For example, transformation induced by the activating protein (DCN-EYFP). Using a combination of biochemical and quantitative approaches, we have shown that decorin evokes a protracted internalization and degradation of the EGFR primarily via caveolar-mediated endocytosis. In contrast to EGF, decorin targets the EGFR to caveolae, but not to early or recycling endosomes. Ultimately, however, both EGF- and decorin-induced pathways converge into late endosomes/lysosomes for final degradation. Thus, we have discovered a novel biological mechanism for decorin that could explain its anti-proliferative and anti-oncogenic mode of action.

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EXPERIMENTAL PROCEDURES

Cells and Materials—A431 human squamous carcinoma cells were obtained from ATCC (Manassas, VA). Dulbecco’s modified Eagle’s medium, fetal bovine serum, 100 times antibiotic-antimycotic solution,
and Dulbecco’s phosphate-buffered saline (DPBS) were purchased from Mediatech (Herndon, VA). All chemicals, unless otherwise specified, were purchased from Sigma. Recombinant human EGFR was purchased from Promega (Madison, WI). Tetramethylrhodamine-conjugated EGFR (EGF-Rhodamine) was purchased from Molecular Probes Inc. (Eugene, OR). Recombinant PhuYFP fluorescent protein was obtained from Evrogen (Moscow, Russia); bis-(sulfosuccinimidyl) suberate and sulfosuccinimidyl-4-(p-maleimidoophenyl)butyrate (Sulfo-SMPB) were purchased from Pierce. Solutions of cross-linkers were freshly prepared before each experiment. Nitrocellulose membrane was purchased from Bio-Rad. Methyl-β-cyclodextrin, filipin, and chlorpromazine were purchased from Sigma. Antibodies include polyclonal rabbit antibodies recognizing the N-terminal region of decorin (37), the C-terminal region of the EGFR (sc-03; Santa Cruz Biotechnology, Santa Cruz, CA), caveolin-1 (BD Transduction Laboratories, San Diego, CA), and monoclonal antibodies recognizing β-actin (Sigma), phospho-pY (PY20; BD Transduction Laboratories), EGF-R (Ab-12; NeoMarkers Inc., Union City, CA), early endosome antigen 1 (EEA1), human CD63, Rab11, and p230 trans-Golgi (all from BD Transduction Laboratories); Rhodamine- or fluorescein isothiocyanate-conjugated anti-mouse and anti-rabbit IgG were purchased from Santa Cruz Biotechnolog; Cy5-conjugated anti-mouse and anti-rabbit IgG were from Chemicon (Temecula, CA). Horseradish peroxidase-conjugated donkey anti-rabbit and sheep anti-mouse were purchased from Amersham Biosciences. SuperSignal West Pico chemiluminescent substrate was purchased from Pierce.

**Plasmid Construction and Transfection**—The enhanced YFP (pRES-EYFP, Clontech) gene was fused to the C terminus of the human decorin sequence obtained from pDCN1 (38) by standard recombinant techniques. Briefly, the pRES-EYFP plasmid was used as a template for PCR amplification using the following synthetic oligonucleotide primers: upstream primer with a XhoI site (underlined), 5′-CGGCCCTGCAGATGGTGAGCAAGGCGAGGA-3′; downstream primer with a BamHI site (underlined) and a stop codon (bold), 5′-CCGGGATCCCGCATCCTACTTGTCAGCTCGTCATGC-3′. The 729-bp PCR fragment was ligated into the pCPE-Pu mammalian expression vector (39, 40) using XhoI and BamHI restriction sites to create the pCPE-Pu-EYFP vector. pDCN1 was used as a template for PCR amplification with the following primers: upstream primer with a KpnI site (underlined) before the insulin signal peptide sequence, 5′-GATGCGCCT-3′; downstream primer with a XhoI site (underlined), 5′-CGGGGAATCTTATAGTTTCCGAGTTGAATGG-3′. The 1113-bp PCR fragment was ligated into the pCPE-Pu-EYFP vector using KpnI and XhoI restriction sites to create the pCPE-Pu-DCN-EYFP vector. The construct was fully sequenced. Human embryonic kidney (293-EBNA) cells were transfected with pCPE-Pu-DCN-EYFP using Lipofectamine (Life Technologies, Inc.). Stable transfectants were obtained by selection with G418 and puromycin (250 and 500 µg/ml, respectively). Positive clones expressing high levels of DCN-EYFP were confirmed by detecting endogenous fluorescence and by Western immunoblotting.

**Expression and Purification of Recombinant Decorin and DCN-EYFP**—Recombinant decorin and DCN-EYFP were produced using either a recombinant vaccinia virus expression system or a stably transfected 293-EBNA cell line. Generation of the recombinant vaccinia virus encoding the mature form of decorin (38) and decorin purification have been described previously (41). DCN-EYFP-expressing 293-EBNA cells were grown to saturation in a CELLine AD 1000 Bioreactor (IBS Integra Biosciences AG, Switzerland), and protein production was achieved by switching to serum-free medium. Details of protein production, purification, and collection are the same as previously published for decorin (41).

**RESULTS AND DISCUSSION**

Decorin Causes a Decrease in EGF-independent (Preformed) EGFR Dimers as Well as EGFR Monomers—It is well established that EGFR dimers represent the active subpopulation of this receptor tyrosine kinase family (35, 42, 43). Upon EGF binding, EGFR monomers dimerize, leading to autophosphorylation of various tyrosine residues within the cytoplasmic domain, and subsequent activation of the signal transduction cascade. We investigated whether decorin would affect EGFR dimerization in the well studied A431 human squamous carcinoma cells, which overexpress wild type EGFR. Decorin has been shown to be effective on these cells both in vitro, by down-regulating EGFR phosphorylation (11), and in vivo, by retarding the growth of A431 tumor xenografts (6, 30). Cells were exposed to recombinant decorin protein

**Cross-linking Experiments**—Subconfluent A431 cells were plated on 4-well chamber slides (Nalge Nunc, Naperville, IL) and grown for 48 h in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum at 37 °C. Cells were washed once with warm serum-free medium and then incubated with DCN-EYFP, DCN, EGF-Rhodamine, or EGF in serum-free medium at 37 °C. Concentrations and incubation times are detailed in the figure legends. Slides were rinsed twice with DPBS and fixed with ice-cold 4% paraformaldehyde in DPBS for 20 min. Cells were permeabilized with DPBS, 0.25% Triton X-100 for 2 min and washed with DPBS, 1% bovine serum albumin. Subsequently, slides were subjected to standard immunofluorescence protocols, and mounted with Vectashield medium (Vector Laboratories Inc., Burlingame, CA). Images were acquired using an Olympus BX51 microscope driven by SPOT Advanced version 4.0.9 imaging software (Diagnostic Instruments Inc.), and a Zeiss Axioplan 200M microscope (Zeiss LSM 5, Carl Zeiss Inc., Oberkochen, Germany). Metamorph 5.0 imaging software (Universal Imaging Corp.) was used for image acquisition and analysis. All dual- or triple-labeled samples were analyzed using filters set at 484 (YFP), 555 (rhodamine), and 620 nm (Cy5). Confocal analysis was performed on a Zeiss LSM 510 META laser scanning confocal microscopy system (Carl Zeiss Inc.) driven by the Zeiss LSM 510 image software. Beam splitters were set at 405 (4,6-diamidino-2-phenylindole), 488 (YFP), 546 (rhodamine), and 633 nm (Cy5). All the images were analyzed using the software mentioned above along with Adobe Photoshop (version 9.0, Adobe Systems, San Jose, CA).

**Image Quantification**—Collected images were analyzed using Adobe Photoshop. Briefly, images of single cells were isolated, and total fluorescent signal was quantified for individual fluorescence channels using the Threshold and Histogram tools. Single cells were then operationally divided into three regions of interest: 1) plasma membrane; 2) intracellular region; and 3) perinuclear region (nucleus included). These areas were subsequently masked and each region of interest was individually quantified. Results were plotted as a percentage of total signals.
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Decorin Causes a Slow and Progressive EGFR Degradation—A plausible interpretation of the results presented above is that decorin affects the equilibrium of ligand-independent EGFR population by decreasing the amount of dimers and monomers via intracellular degradation. To further investigate this possibility, we incubated A431 cells with decorin core or EGF for the same time points as above (0.5–6 h) but without any cross-linking reagent. Surprisingly, total EGFR did not rapidly decline upon incubation with the decorin core (Fig. 2A). In the constant presence of decorin, total EGFR levels declined slowly during the first 2 h of incubation and later on began to drop significantly, reaching a plateau at ~3 h (Fig. 2B). Notably, the presence of exogenous decorin core led to a protracted reduction by ~40% of total EGFR content that lasted for up to 6 h. In contrast, EGF treatment caused a significant and faster decline in total EGFR followed by a nearly full recovery (Fig. 2, A and B). The decorin-induced reduction in EGFR is in agreement with the estimates derived from A431 clones stably expressing decorin. In this study we found three individual clones with ~40% EGFR levels of control wild-type cells (11). In addition, we found that the average number of $^{125}$I-EGF binding sites in three independent clones expressing decorin was $1.3 \times 10^6 \pm 0.2$ receptors per cell as compared with $2.4 \times 10^6 \pm 0.1$ receptors per cells in the wild-type A431 (11). The dissociation constants determined by Scatchard analysis were not significantly different from those found in control cells (11).

core (0.67 μM) or EGF (16 nM) for various intervals and then treated for the last 20 min with the membrane-impermeable chemical cross-linker Sulfo-SMPB (0.5 mM) (41). Decorin protein core caused a reduction in the amount of both monomers and dimers (Fig. 1A). Quantitative analysis of three independent experiments showed that decorin protein core caused a significant and progressive decline in the amount of ligand-independent (preformed) dimers, with a $t_{1/2}$ of ~45 min (Fig. 1B). The EGFR dimer levels reached a plateau at 1 h (70% reduction) and this did not change for up to 6 h. Interestingly, the EGFR monomer amount also declined with similar kinetics; however, its levels partially recovered and reached a plateau at later time points remaining at ~60% of control EGFR levels (Fig. 1C). Comparable results were obtained using recombinant decorin proteoglycan (not shown). In contrast, EGF caused a rapid ($t_{1/2} \sim 2$ min) and substantial (~7-fold) increase in EGFR dimers, and this did not appreciably change at later time points (Fig. 1D). In the presence of EGF, the amount of EGFR monomers declined in the first 30 min, but subsequently increased and thereafter remained relatively constant at ~90% of control levels (Fig. 1E). These data indicate that decorin protein core, independent of its glycosaminoglycan moiety, affects the intracellular fate of EGFR monomers and dimers in a way that differs from EGF.
Among the control and decorin-expressing A431 cells, indicating that decorin did not appreciably change EGFR affinity for EGF. Collectively, these results indicate that decorin induces EGFR degradation via a slow pathway that, in contrast to that evoked by EGF, does not lead to recovery.

Decorin Can Be Cross-linked to the EGFR and Forms Large Supramolecular Aggregates—After comparing the results shown in Fig. 2, A and B, with the cross-linking data shown in Fig. 1, an apparent discrepancy was noted in the amount of total EGFR detected upon decorin core treatment, especially at the earlier time points, 30 min and 1 h (compare Figs. 1C and 2B). Upon cross-linking and Western immunoblotting analyses, ~50% of total EGFR was unaccounted for. These data suggest that in the cross-linking experiments a proportion of the EGFR could not be detected, possibly because it was migrating in larger polymeric complexes, which either did not penetrate the 3–15% gradient SDS-PAGE or did not efficiently transfer onto the membrane. Cross-linking experiments were repeated and, indeed, a longer exposure revealed an EGFR immunoreactive band migrating slower than the EGFR dimer (Fig. 2C, arrow). It is well known that EGFR exists in a monomeric form (175 kDa) and in several aggregated states ranging between 350 and >500 kDa (44). Thus, to determine whether decorin core could also be a component of EGFR-containing high molecular mass complexes, we probed the high molecular mass region (>350 kDa) with an anti-decorin antibody. A significant amount of decorin was cross-linked with EGFR dimers (Fig. 2D, arrow), and higher order oligomers. These decorin-containing aggregates were not present in the absence of decorin or when the cells were challenged with EGF (Fig. 2D), indicating specificity of the decorin signal. Therefore, our results show for the first time a covalent cross-linking between decorin protein core and EGFR dimers and oligomers. Considering that the cross-linker used (Sulfo-SMPB) has an arm length of 14.5 Å, decorin core had to be in very close proximity to the ligand-binding region of the EGFR. By plotting the relative mobility of protein standards from several blots, we estimated the molecular mass of cross-linked decorin-EGFR complexes to be ~450 kDa (Fig. 2D, arrow). This would correspond to an EGFR dimer (~350 kDa) and two decorin core molecules (~100 kDa), suggesting a stoichiometry of 1:1 decorin core:EGFR monomer. These data provide an explanation for the apparent loss of EGFR after cross-linking in the presence of decorin core. Most likely, EGFR epitopes would be masked in the immunoblots by the formation of EGFR-decorin complexes. Our results, however, cannot rule out the presence of other cross-linked cell surface proteins in addition to decorin and EGFR in the larger supramolecular aggregates with molecular mass >500 kDa.

Decorin Inhibits EGF-dependent EGFR Dimerization—The previous results raised the possibility that decorin could directly interact with the EGFR by forming supramolecular complexes. Previous studies using the yeast two-hybrid system have shown that decorin binds to the EGFR within its ligand-binding L2 domain (36). The binding site partially overlaps with but is distinct from the EGF-binding domain. Thus, we determined whether decorin would compete with EGF in inducing the initial step of EGFR activation, that is, receptor dimerization. In these experiments the cells were first challenged with decorin core (0.6 μM) for either 2 or 5 min and then with EGF (16 or 8 nM) for 10 min. After incubation, cells were exposed to the impermeable cross-linker bis(sulfosuccinimidyl) suberate (0.5 mM). A representative immunoblotting is shown in Fig. 3A. Densitometric analysis of the immunoblots revealed that EGFR dimer was reduced by ~30 and 20% at 8 and 16 nM EGF, respectively, with an average ~25% inhibition (Fig. 3B). In another set of experiments, cells were pretreated with decorin core (0.6 μM) for 30 min and then with EGF (8 or 16 nM) for 10 min. In this case, there was 30 and 70%, respectively, inhibition of EGFR dimer formation (Fig. 3C). Collectively, these results indicate that decorin inhibits EGF-dependent EGFR dimerization in a manner dependent on EGF concentration. These data raise the possibility that decorin could compete with traditional ligands, such as EGF or TGF-α, for their binding to EGFR. This might represent an additional mechanism through which decorin could control tumor cell growth.

DCN-EYFP Induces EGFR Internalization and Partially Co-localizes with the Receptor in Intracellular Vesicles—To further investigate the role of decorin in EGFR internalization, we generated a chimeric pro-
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**FIGURE 3.** Decorin inhibits EGF-dependent EGFR dimerization. A, representative immunoblotting showing EGFR dimer formation in quiescent A431 cells after incubation with EGF alone (8 nm) or with decorin core (0.57 μM) and EGF. Cells were preincubated with decorin for 5 min, and then with EGF for 10 min. Subsequently, the cells were incubated with the cross-linker bis-(sulfosuccinimidyl) suberate (BS3), 0.5 mM, for 30 min at room temperature. Total cell lysates were separated on a 3–15% linear SDS-PAGE and immunoblotted to detect EGFR. B, quantification of competition and cross-linking experiments (mean ± S.E., n = 3). Note the significant inhibition (p < 0.05, asterisk) of EGFR dimer formation with cells incubated with EGF in the presence of decorin. C, Western immunoblotting of competition and cross-linking experiments. Cells were preincubated with decorin (0.57 μM) for a longer time (30 min) before the addition of EGF (8 or 1.6 nm) for 10 min. Cross-linking reaction was carried out using 0.5 mM Sulfo-SMPB for 20 min at 37 °C. Note the marked suppression of EGFR dimerization evoked by decorin, especially at the lower (1.6 nm) EGF concentration.

**FIGURE 4.** Creation of a functional decorin-EYFP chimera. A, schematic diagram of the construct used to generate the decorin-EYFP consisting of an insulin signal peptide (Insulin) and a His tag (HisX6) for purification, both upstream of the mature form of the decorin sequence (DCN). The EYFP sequence was fused to the C terminus. Appropriate restriction sites are also shown. B, 293-EBNA cells stably transfected with pCEP-Pu-DCN-EYFP (clone DY8) shown by fluorescence microscopy in the presence of the EYFP signal (Fig. 4A, upper panel). These vesicles became larger around the nucleus and showed EGFR surrounding DCN-EYFP, forming a “doughnut-shaped” structure (Fig. 5B, bottom panels, arrows). These results suggest the possibility that the EGFR might still be intercalated in the membrane of the endocytic vesicles, whereas decorin is located within their lumina.
Similar results were obtained by pulsing with DCN-EYFP for 30 min followed by chasing for 10 or 30 min with serum-free medium (not shown).

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Next, we addressed the issue of specificity, insofar as the EYFP moiety, linked to the C terminus of decorin, could have been dissociated from the decorin protein core resulting in a misleading interpretation of our results. To this end, cells that were incubated with DCN-EYFP for 60 min were reacted with an antibody against the N terminus of decorin followed by fluorescence-labeled secondary antibody. Signal merge showed that the pattern of fluorescence using the antibody against decorin was identical to the one from the EYFP channel (Fig. 5C). Note that nonspecific staining, because of the secondary antibody, was present in the nucleus. The fact that the EYFP signal co-localized with decorin led us to conclude that decorin is responsible for the effects shown in Fig. 5. Furthermore, DCN-EYFP appears to be relatively stable after internalization.

To further confirm the issue of specificity, we performed competition experiments by incubating A431 cells with a constant amount of DCN-EYFP (0.4 μM) and 20- and 30-fold molar excess of decorin protein core. The results showed a nearly complete block of binding and internalization when decorin protein core was used, indicating that the major binding was indeed provided by decorin protein moiety (Fig. 6). Finally, we incubated A431 cells with recombinant PhiYFP, a yellow fluorescent protein highly homologous to EYFP, and found no significant binding (not shown). In summary, using DCN-EYFP chimeric proteoglycan and fluorescence microscopy, we show for the first time in living cells that decorin causes a protracted internalization of the EGFR, that this process is mediated by decorin protein core, and that both the receptor and DCN-EYFP co-localize within endocytic vesicles.

**Reduced EGFR Levels Diminish DCN-EYFP and EGF Binding and Endocytosis**—To further test the specificity of the DCN-EYFP/EGFR interaction, we selected A431 clones expressing low EGFR, by chronically exposing them to a high concentration (100 ng/ml) of EGF. These concentrations are known to be toxic for wild-type A431 cells and allow for selection of clones that express low levels of EGFR (47, 48). After several weeks of continuous culture in the presence of EGF, several surviving clones were isolated. Western immunoblotting showed that clones A10 and B6 contained ∼40% of the EGFR expressed by the wild-type cells (Fig. 7A). Wild-type A431 and clone A10 cells were incubated with DCN-EYFP for 30 min at 37 °C or EGF-Rhodamine for 10 min. Binding and internalization of both DCN-EYFP and EGF were significantly reduced in clone A10 (Fig. 7B). Similar results were obtained with clone B6 (not shown). Thus, decorin binding and internalization is quantitatively dependent on EGFR expression in a way similar to EGF. These data represent further evidence that EGFR is a direct partner for decorin binding on the cell surface.

**Decorin-induced EGFR Internalization Does Not Require EGFR Tyrosine Kinase Activity**—In eukaryotic cells, internalization of cell surface receptors and their ligands occurs via clathrin-dependent and -independent pathways (35, 49–51). In general, EGFR movement from caveola to clathrin-coated pits is considered to be the initial event in internalization and signaling (35), and there is evidence that its receptor...
tyrosine kinase activity is required for proper EGFR internalization (52).

Therefore, we tested whether EGFR internalization evoked by decorin would also require EGFR tyrosine kinase activity, by using the tyrphostin AG1478, a highly specific and selective inhibitor of EGFR tyrosine kinase when used at low dosages (53). We have previously shown that incubation of A431 cells with AG1478 (1–5 μM) completely blocked EGF-induced EGFR phosphorylation without affecting platelet-derived growth factor receptor phosphorylation induced by platelet-derived growth factor (9). A431 cells were preincubated with AG1478 (5 μM) for 60 min and then incubated with EGF-Rhodamine (3 nM) for 10 min, followed by a 10-min chase at 37 °C. Cells were fixed, permeabilized, and then incubated with a monoclonal anti-EGFR antibody, followed by a fluorescein isothiocyanate-conjugated secondary antibody. Pretreatment with AG1478, a specific inhibitor of EGFR tyrosine kinase, prevents the internalization of EGF-EGFR complexes (arrows) but not the binding of the EGF to the cell surface. Confocal microscopy images showing that AG1478 inhibits EGF/EGFR endocytosis. A431 cells were preincubated with AG1478 (5 μM) for 60 min and then incubated with DCN-EYFP (0.78 μM) for 30 min, followed by a 30-min chase at 37 °C. Cells were fixed, permeabilized, and processed as in C. Notice that in contrast to EGF, decorin-induced endocytosis of the EGFR is not appreciably inhibited by the tyrphostin (arrows). Scale bars = 10 μm.

In contrast, AG1478 did not appreciably block decorin-induced EGFR internalization. Following a 30-min pulse with decorin-EYFP and a 30-min chase in serum-free medium (longer incubation periods were used because of the slower kinetics of decorin), numerous large vesicles containing both decorin and EGFR were noted; these vesicles were primarily located in the perinuclear area (Fig. 7D, arrows). These results indicate that the EGFR internalization evoked by decorin differs from that induced by EGF, and that EGFR tyrosine kinase is not required for EGFR endocytosis following decorin challenge.

**Decorin Causes Internalization of EGFR via Caveolae**—The previous results raised the possibility that EGFR internalization evoked by decorin could involve a clathrin-independent pathway. Indeed, the association of the EGFR with lipid rafts and caveolae can affect receptor sequestration and trafficking in a number of cells (55–58). Caveolae are cholesterol-rich, detergent-insoluble microdomains of the plasma membrane that are enriched in cell surface receptors, such as EGFR (58), although in A431 cells EGFR may not be concentrated in caveolae but rather randomly distributed in the plasma membrane (59). Interestingly, AG1478-mediated inhibition of EGFR tyrosine phosphorylation in glioblastoma cells increases receptor association with caveolae (57).
Thus, we first investigated whether exogenous decorin would cause a redistribution of caveolin-1, one of the major integral caveolar proteins that functions as a scaffolding matrix to organize and concentrate numerous signaling and adaptor molecules within caveolae (58). A431 cells were treated with DCN-EYFP at 37 °C for different time points. At time 0, most caveolin-1 was present in the perinuclear area, presumably in the trans-Golgi network, and some was diffusely distributed on the plasma membrane and cytoplasm as small scattered spots (Fig. 8A). After incubation with DCN-EYFP for 15 min, most caveolin-1 migrated to the inner side of the plasma membrane (Fig. 8A, 15 min, arrows) and

FIGURE 8. Decorin causes internalization of the EGFR via caveolae. A, fluorescence microscopy images of A431 cells incubated with DCN-EYFP (0.78 μM) for 15, 30, and 60 min at 37 °C and stained for caveolin-1, a major component of caveolae. Cells were fixed, permeabilized, and then incubated with a polyclonal anti-caveolin-1 antibody followed by a rhodamine-conjugated secondary antibody. Colors were adjusted by using Adobe Photoshop. Arrows indicate the plasma membrane, whereas arrowheads show caveolin-positive vesicles. B, multiple images obtained from experiments as in panel A were used to quantify the percent distribution of caveolin signal on the cell surface, intracellular, and perinuclear areas, as indicated. Data represent mean values ± S.E. of images collected from six cells/time point. C, confocal microscopy images showing partial co-localization of EGFR, caveolin-1, and decorin. A431 cells were incubated with DCN-EYFP for 30 min, chased for 30 min, and then treated as described before. Monoclonal anti-EGFR and polyclonal anti-caveolin antibodies were used. Merged image shows co-localization of EGFR, caveolin, and DCN-EYFP signals (arrows) in the perinuclear area. D, magnification of the merged image in C with the addition of 4,6-diamidino-2-phenylindole staining. Vesicles in which EGFR, caveolin, and DCN-EYFP signals co-localize (white dots) are indicated by the horizontal arrows. Nu, nucleus. Go, Golgi. Scale bars = 10 μm.
some accumulated to form larger spots (arrowheads). After 30 min, large vesicles were internalized and began accumulating in the perinuclear region (Fig. 8A, 30 min, arrowheads). After 60 min, most vesicles accumulated in the perinuclear area, few were located in the cytoplasm, and even fewer were associated with the plasma membrane. Quantification of the changes in caveolin-1 distribution showed a rapid decline (15 min) of the perinuclear caveolin-1-rich endocytic vesicles and a concurrent enrichment of cell surface vesicles (Fig. 8B). Within the next 45 min, the distribution of the perinuclear and cell surface caveolin-1-positive vesicles returned to nearly basal levels. In contrast, the intracellular (i.e., cytoplasmic) fraction of the caveolin-positive vesicles did not appreciably change with time (Fig. 8B). In summary, decorin treatment causes a rapid (15 min) displacement of caveolin-1 from the trans-Golgi area to the plasma membrane, followed by formation of large caveolin-positive structures inside the cell. These results support the concept that decorin-induced caveolin-1 redistribution might be closely linked to the EGFR internalization pathway. Indeed, confocal microscopy analysis showed partial, but significant, co-localization of DCN-EYFP, EGFR, and caveolin-1 in the perinuclear area (Fig. 8, C and D, arrows). In contrast, little or no co-localization of EGFR with caveolin-positive vesicles was observed when EGF was used (not shown). The results clearly show that the endocytic vesicles containing decorin and EGFR are enriched in caveolin-1 and are located in the perinuclear region.

Cholesterol Depletion Affects Decorin-induced EGFR Internalization—
To further address the role of caveola in the internalization of decorin-EGFR complexes, we utilized methyl-β-cyclodextrin (MβCD). MβCD is a water-soluble cyclic heptasaccharide that binds cholesterol with high specificity and, because of its ability to deplete plasma membrane cholesterol, has been utilized as an agent to disrupt membrane rafts (60). Caveola formation on the cell surface is critically dependent on the presence of cholesterol and its compartmentalization. Therefore, in the presence of MβCD, caveolin-dependent endocytosis can be selectively inhibited (51). Interestingly, MβCD triggers ligand-independent activation of the EGFR in several cells including A431 cells (57, 59, 61). Utilizing relatively low concentrations of MβCD (2.5 mM) (higher concentrations, 5 and 10 mM, were found to be toxic to the cells) we discovered that, in contrast to EGF, decorin-induced internalization of the EGFR was markedly blocked by the drug (Fig. 9). Following a 30-min incubation in the presence of MβCD, most of decorin-EGFR complexes were located along the plasma membrane (Fig. 8, lower middle panel); in contrast, following a 10-min incubation with EGF, the EGF-EGFR complexes were properly internalized and reached perinuclear vesicles (Fig. 9, top middle panel).

To further investigate decorin-induced caveolae-mediated EGFR internalization, we used the drug filipin, which is known to bind and sequester cholesterol in the plasma membrane and impair the invagination of caveolae, thereby inhibiting their internalization (62, 63). Pretreatment of cells with 5 μg/ml filipin for 60 min blocked endocytosis of both EGF-Rhodamine and DCN-EYFP, without blocking the binding to EGFR on the cell surface (not shown). When we lowered the filipin concentration to 2 μg/ml (30 min pretreatment), we obtained a result comparable with MβCD. DCN-EYFP co-localized with EGFR on the plasma membrane and the decorin-EGFR complexes were not internalized. In contrast, EGF-induced EGFR endocytosis was not affected (not shown).

Decorin-induced Internalization of the EGFR Is Not Affected by Chlorpromazine—Next, we tested the effects of chlorpromazine, a cationic amphiphilic drug that prevents the assembly of clathrin adapter protein AP-2 on clathrin-coated pits, and also blocks receptor recycling (64). Chlorpromazine causes the loss of coated pits from the surface of human fibroblasts and the concurrent appearance of clathrin coats on endosomal membranes (64). In A431 cells, the internalization of EGF-EGFR complexes was markedly reduced by 30 min incubation with relatively low dosages (7.5 μM) of chlorpromazine (Fig. 9, top right panel). Higher concentrations (15 and 30 μM) of the drug induced significant morphological changes and, therefore, were not utilized. In contrast, the decorin-EGFR complexes were properly internalized and reached the perinuclear region within 30 min of incubation (Fig. 9, bottom right panel). Collectively, these results support the concept that cholesterol-rich membrane microdomains and caveolae are necessary for proper EGFR endocytosis induced by decorin, and that this pathway, in contrast to EGF, does not significantly involve clathrin-coated pits.

Decorin Does Not Target the EGFR to Either Recycling Endosomes or the Golgi—It has been reported that caveola-mediated endocytosis can directly target the vesicles to the Golgi apparatus (65). Therefore, we tested whether decorin would target EGFR to the Golgi apparatus and then to secretory vesicles for recycling to the cell surface. A431 cells were incubated with EGF (3 nM) or DCN-EYFP (0.78 μM) for 10 and 30 min, respectively, at 37 °C, and then chased for various time points.

The cells were then reacted with antibodies directed against EGFR and Rab11, a 24-kDa GTPase originally isolated from the Golgi-microsomal
Decorin Causes EGFR Internalization via Caveolae

FIGURE 10. Decorin does not target the EGFR to either recycling endosomes or the Golgi. A and B, confocal microscopy images of A431 cells incubated with EGF (3 nM) or DCN-EYFP (0.78 μM) for the times indicated at 37 °C. Immunofluorescence was performed using antibodies against the EGFR or Rab11, a marker of recycling endosomes, followed by appropriate secondary antibodies. EGFR and Rab11 co-localization is shown in the merged image (orange-yellow). Insets: higher magnification of areas in the white square. C, confocal microscopy showing lack of DCN-EYFP localization in the Golgi apparatus. A431 cells were incubated with DCN-EYFP (0.78 μM) for the times indicated. To visualize the Golgi we used a monoclonal antibody against p230, a specific marker for the trans-Golgi network, followed by 4,6-diamidino-2-phenylindole (DAPI) staining. Images represent sequential sections along the z axis (1.74, 2.18, 3.49, 3.92 and 4.79 μm from left to right, respectively). Notice the lack of co-localization of endocytosed decorin within the Golgi even after 2 h. Scale bars = 5 μm.

FIGURE 11. Internalized EGFR induced by decorin does not localize to early endosomes. A and B, confocal microscopy images of A431 cells incubated with EGF-Rhodamine or DCN-EYFP (same concentrations as in Fig. 9) for the times indicated. Cells were immunostained for EGFR (red) or EEA1 (green), a marker for early endosomes. Fluorescent EGFR-Rhodamine or DCN-EYFP were detected using appropriate filters. The merged image shows co-localization vesicles (yellow). Insets: high magnification images of the areas in the white rectangles. Both EGF and EGFR extensively co-localize with EEA1+ endosomes (A), in contrast to decorin-EGFR complexes, which only minimally co-localize with EEA1+ endosomes (B). Nu, nucleus. Scale bars = 10 μm.

fraction and commonly used as a marker for both trans-Golgi network and recycling endosomes (66). Confocal microscopy revealed that EGF induced extensive localization of EGFR into the recycling Rab11+ compartment (Fig. 10A). In contrast, DCN-EYFP evoked no significant co-localization of EGFR and Rab11 (Fig. 10B). These findings were further corroborated by the observation that DCN-EYFP did not co-localize with the protein p230 (67), a specific marker for the trans-Golgi network (Fig. 10C). This is a key compartment for the endocytic/secretory pathway from which proteins are sorted either for constitutive transport to the late endosomes or for recycling to the cell surface. Thus, decorin targets the EGFR to a compartment other than the Golgi apparatus (most likely the “caveosome,” see below) and does not cause significant recycling of the receptor to the cell surface, even after a 2-h, 30-min pulse and 90-min chase (not shown).

Internalized EGFR Induced by Decorin Does Not Localize to Early Endosomes—To further investigate the decorin-induced EGFR endocytosis pathway, we tested for the presence of EGF-Rhodamine-EGFR and DCN-EYFP-EGFR complexes in early endosomes. To label early endosomes, we used an antibody against EEA1, a 180-kDa peripheral membrane protein that resides within early endosomes (68). Typically, entrance to EEA1+ compartments occurs through clathrin-mediated endocytosis (68), and transmembrane proteins in the EEA1+ compartment can either enter the late endosomes/lysosomes for degradation or the Rab11+ recycling endosomes (69). A431 cells were incubated with EGF-Rhodamine (3 nM) for 10 min and chased for 10 min, or DCN-EYFP (0.78 μM) for 30 min followed by a 30-min chase. Confocal microscopy revealed that both EGF-Rhodamine and EGFR largely co-localized within EEA1+ endosomes (Fig. 11A, merge). This is in line with the fact that EGF remains associated with the endocytosed EGFR down to late endosomes, and that it is released from the receptor at very low pH within lysosomes (35). In contrast, DCN-EYFP and the internalized EGFR evoked by DCN-EYFP only minimally localized within EEA1+ vesicles (Fig. 11B). This corroborates our finding presented above that DCN-EYFP does not appreciably target the endocytosed EGFR to the Rab11+ recycling compartment (Fig. 10B).

Decorin and Late Endosomes—Next, we investigated whether decorin-induced EGFR internalization would lead to late endosome localization, and subsequently to receptor degradation. As a marker for the late endosome/lysosome compartment, we used an antibody against CD63, a 53-kDa type III lysosomal glycoprotein (also known as gp55). CD63 is a member of the tetraspan transmembrane 4 superfamily and has been previously described to be a specific protein of the late endosome/lysosome compartment (70). Following a 30-min incubation and 90-min chase, DCN-EYFP induced a significant co-localization of EGFR within CD63+ endosomes, in a manner comparable with that evoked by EGF-Rhodamine (Fig. 12). Further confocal microscopy analysis showed that both EGF-Rhodamine and DCN-EYFP were also present in late endosomes, presumably targeted for final degradation in the lysosomes (not shown). These results indicate that decorin evokes a protracted down-regulation of the EGFR via a caveosome/late endosome/lysosome axis and provide a plausible explanation for the reduced EGFR.
content in A431 cells exposed to recombinant decorin or when decorin is endogenously produced by transgenic experiments (11).

Conclusions—In this study, we report a novel mechanism of action induced by a soluble proteoglycan, decorin, in controlling EGFR, an important receptor tyrosine kinase widely implicated in the pathogenesis and progression of cancer. Collectively, our results demonstrate that decorin is internalized through a unique endocytic pathway that is qualitatively and topographically different from that induced by EGF in the same in vitro system. A working model summarizing the present results is provided in Fig. 13. Accordingly, decorin causes a slow and sustained internalization of the EGFR primarily via caveolae-mediated endocytosis. Decorin targets the EGFR to a distinct intracellular compartment, most likely the “caveosome,” because decorin-induced EGFR internalization does not appreciably involve EEA1⁺ early or Rab11⁺ recycling endosomes. Eventually, EGFR is transferred to the CD63⁺ late endosome compartment, presumably for final degradation by acid hydrolases. In contrast, EGF induces the canonical and well-established EGFR endocytosis primarily via the clathrin-dependent pathway that utilizes EEA1⁺ early endosomes and finally CD63⁺ late endosome/lysosomes. Some internalized EGFR can then be recycled to the plasma membrane via Rab11⁺ endosomes.

We note that decorin causes an inhibition of EGFR phosphorylation (11) in addition to receptor down-regulation. This is noteworthy because a low phosphorylation state of the EGF receptor is associated with increased receptor localization to caveolae and attenuation of the transformed phenotype (57). Thus, strategies that induce EGFR association with caveolae may be beneficial in suppressing aberrant signaling events in various tumor systems. Our findings confirm and expand this concept and further stress that caveolar-mediated endocytosis of the EGFR, and perhaps other receptors, can be an additional anti-oncogenic mechanism that could be operational in vivo. The recent discovery that decorin also signals via the insulin-like growth factor receptor (7) suggests that this proteoglycan may have a broader bioactivity.

There are two recent examples of caveolar involvement in receptor control. The first is provided by the transforming growth factor-β (TGF-β) receptor, which utilizes both caveolae and clathrin-coated pits for its internalization (71). Notably, the latter pathway promotes TGF-β signaling, whereas the former pathway leads to receptor degradation, as in the case of decorin. Thus, segregation of the TGF-β receptor within distinct endocytic compartments regulates TGF-β/Smad signaling and receptor turnover. The second example is provided by the EGFR, which can be diverted to either clathrin- or caveolar-dependent endocytosis based on the amount of exogenous EGF. At low (0.25 nM) EGF concent
