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Epidermal growth factor (EGF)-like repeats of human tenascin-C as ligands for EGF receptor

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Signaling through growth factor receptors controls such diverse cell functions as proliferation, migration, and differentiation. A critical question has been how the activation of these receptors is regulated. Most, if not all, of the known ligands for these receptors are soluble factors. However, as matrix components are highly tissue-specific and change during development and pathology, it has been suggested that select growth factor receptors might be stimulated by binding to matrix components. Herein, we describe a new class of ligand for the epidermal growth factor (EGF) receptor (EGFR) found within the EGF-like repeats of tenascin-C, an antiadhesive matrix component present during organogenesis, development, and wound repair. Select EGF-like repeats of tenascin-C elicited mitogenesis and EGFR autophosphorylation in an EGFR-dependent manner. Micromolar concentrations of EGF-like repeats induced EGFR autophosphorylation and activated extracellular signal–regulated, mitogen-activated protein kinase to levels comparable to those induced by subsaturating levels of known EGFR ligands. EGFR-dependent adhesion was noted when the ligands were tethered to inert beads, simulating the physiologically relevant presentation of tenascin-C as hexabrachion, and suggesting an increase in avidity similar to that seen for integrin ligands upon surface binding. Specific binding to EGFR was further established by immunofluorescence detection of EGF-like repeats bound to cells and cross-linking of EGFR with the repeats. Both of these interactions were abolished upon competition by EGF and enhanced by dimerization of the EGF-like repeat. Such low affinity behavior would be expected for a matrix-"tethered" ligand; i.e., a ligand which acts from the matrix, presented continuously to cell surface EGF receptors, because it can neither diffuse away nor be internalized and degraded. These data identify a new class of "insoluble" growth factor ligands and a novel mode of activation for growth factor receptors.

Introduction

The EGF receptor (EGFR)* transduces signals from the extracellular milieu to trigger diverse cell functions. EGFR signaling has been shown to play critical roles in organogenesis, tissue maintenance and repair, and, when dysregulated in cancers, to promote tumor progression (Khazaie et al., 1993; Wells et al., 1998; Wells, 2000). The several known ligands for EGFR are peptide growth factors that are processed from membrane-associated propeptides. These ligands can engage and activate EGFR in both their soluble and membrane-anchored forms (Brachmann et al., 1989; Anklesaria et al., 1990; Wells et al., 1990) and when chemically tethered to a substratum (Kuhl and Griffith-Cima, 1996). In addition, EGFR signaling and mitogenesis can be fully signaled from plasma membrane–restricted receptors (Wells et al., 1990; Vieira et al., 1996; Haugh et al., 1999). As internalization of ligand serves to attenuate signaling (Wells et al., 1990; Reddy et al., 1996b), presenting an EGFR ligand as part of a larger extracellular complex would present novel signaling and modulatory possibilities. It may, for example, serve to coposition activated integrin and growth factor receptors to modulate integrin functioning in processes like cell migration. Although growth factor receptors have been traditionally thought to interact with high avidity to individual ligands, the discoidin domain receptor recognize and initiate signaling in response to epitopes in matrix collagen (Shrivastava et al., 1997; Vogel

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*Abbreviations used in this paper: EGFR, EGF receptor; ERK, extracellular signal–regulated kinase; M, mutant; MAP, mitogen-activated protein; mEGF, murine EGF; PEO, polyethylene oxide; WT, wild-type.

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et al., 1997). Thus, it has been hypothesized that similar matrix signals may exist for other growth factor receptors.

Several matrix components possess EGF-like repeats, the functions of which are largely unknown. Two of these components have been suggested to initiate signaling through the EGFR. The EGF-like repeats in laminin and tenascin-C (hexabrachion) have been shown to modulate cell adhesion and cell motility (Prieto et al., 1992; Nelson et al., 1995). Experiments have suggested that these repeats may directly trigger EGFR signaling by acting as very low affinity ligands (Engel, 1989; Panayotou et al., 1989; Nelson et al., 1995) or potentiate signaling from soluble EGF (Jones et al., 1997). However, these previous studies did not directly isolate EGFR signaling or binding and therefore the exact mechanism of signaling remains undetermined. Furthermore, low affinity ligands (dissociation constant kd in the micromolar range at best) would not be detected by standard binding assays. Low values of solution phase affinity would be predicted for matrix-embedded EGFR ligands because they effectively act from two dimensions, constrained at the cell surface. The effective concentration is increased by being constrained to the interface between the extracellular matrix and the cell surface. Further, the tethered ligand receptor complexes are physically restrained from entering the cell and thus impervious to the major long-term attenuation mechanism of ligand-dependent internalization and degradation (Herbst et al., 1994).

We decided to investigate whether the EGF-like repeats in tenascin-C activate EGFR, as tenascin-C is restricted to sites of tissue development and regeneration and is up-regulated in tumor cells, all of which are sites of EGFR functioning (Erickson, 1993; Chiquet-Ehrismann, 1995). To isolate EGFR signaling, we used NR6 mouse fibroblasts devoid of endogenous EGFR (Pruss and Herschman, 1977) that have been engineered to express various EGFR constructs (Wells, et al., 1990). We found that select EGF-like repeats of tenascin-C were capable of eliciting mitogenesis in an EGFR-dependent manner. Furthermore, although EGFR autophosphorylation was negligible at best, extracellular signal–regulated kinase (ERK) mitogen-activated protein (MAP) kinase activation was comparable to subsaturating levels of known EGFR ligands. These cell responses required the extracellular ligand-binding motifs of EGFR, suggesting direct binding. EGFR-dependent adhesion was noted when the predicted avidity of the EGF-like repeat was increased by dimerization or polyvalency via tethering the ligands to inert beads, thereby simulating the physiologically relevant presentation of tenascin-C as hexabrachion. Immunofluorescent imaging further revealed EGFR-dependent binding of the EGF-like repeats to the cell surface. These bindings were abolished upon preincubation with EGF ligand. Direct proof of interaction was demonstrated by cross-linking of EGFR to the EGF-like repeats of tenascin-C.

Results

EGF-like repeats of tenascin-C have mitogenic activity dependent on functional EGFR

The EGF-like repeats of tenascin-C were expressed and purified as recombinant proteins in *Escherichia coli*; this mode of expression was chosen because bacterial expression results in fully functional EGF (Reddy et al., 1996a). All 14 repeats were divided among separate clones containing one, two, or three contiguous repeats. Each clone was tested for EGFR-dependent mitogenic activity by a ^{3}H-thymidine incorporation assay using NR6 fibroblasts transduced to overexpress either the wild-type (WT) or a kinase-inactive mutant (M) EGFR (WT NR6 and M NR6, respectively). The EGFR M mutant contains a methionine substituted for lysine 721, abolishing the kinase activity of the EGFR and, hence, its autophosphorylation. Cells were treated with concentrations of each protein, which varied from 1–4 uM, depending on the preparation/purification. The two proteins corresponding to EGF-like repeats 1/2 (Ten1/2) and 11/12/13 (Ten11/12/13), exhibited mitogenic activity on WT NR6 cells at 2 and 4 uM, respectively, but not at 10 and 1% of their initial concentration (Fig. 1 A). Ten14 (the 14th EGF-like repeat from the NH_2-terminal end) exhibited mitogenic activity at 3 uM (ninefold over uninduced) and slight mitogenic activity at 0.3 uM (threefold over uninduced), but none at 0.03 uM. Since known soluble EGFR ligands can drive proliferation at ~0.01 kd, this suggested that we were at the lower end of effective ligand concentration and below the solution kd of this matrix-derived ligand (Reddy et al., 1996a). The mitogenic activity was dependent on functional EGFR, as no increased incorporation was observed for the M NR6 cells expressing the kinase-inactive EGFR mutant. To exclude the possibility that these repeats might be potentiating an unknown growth factor signal within the quiescence media, mitogenesis was repeated in serum-free media for the Ten14 repeat. Fold induction of thymidine incorporation over uninduced control in dialyzed serum-free media was comparable to results obtained with quiescence media (Fig. 1 B). Thus, mitogenic stimulation is an inherent property of these repeats. The absence of activity from similar concentrations of proteins containing repeats 9/10, and of EGF-like repeats from laminin β1 chain (data not shown), which were cloned and purified in parallel and identical manner, establishes specificity of the observed activity to select EGF-like repeats.

EGF-like repeats of tenascin-C directly activate EGFR

The ability of these three repeat proteins to activate the EGFR was assessed by induction of tyrosine phosphorylation of the EGFR in treated cells. Activation of the WT EGFR of WT NR6 cells was induced upon treatment with each of the three repeat proteins that scored for mitogenesis, as detected by slightly increased tyrosyl-directed phosphorylation (Fig. 2 A). The induced activation required EGFR kinase, because tyrosine phosphorylation of the kinase-inactive EGFR mutant protein of M NR6 cells was not observed upon treatment. The degree of EGFR phosporylation by the EGF-like repeats was seen to be relatively small compared with EGF or the low-affinity EGF ligand Y13G at concentrations approximately kd (~2 nM for EGF; ~0.1 uM for Y13G); yet it was comparable to the degree of EGFR phosphorylation achieved by lower (~0.01 kd), but physiologically relevant, concentrations of these two ligands (Fig. 3 A). Both EGF and Y13G elicit maximal stimulation of mito-

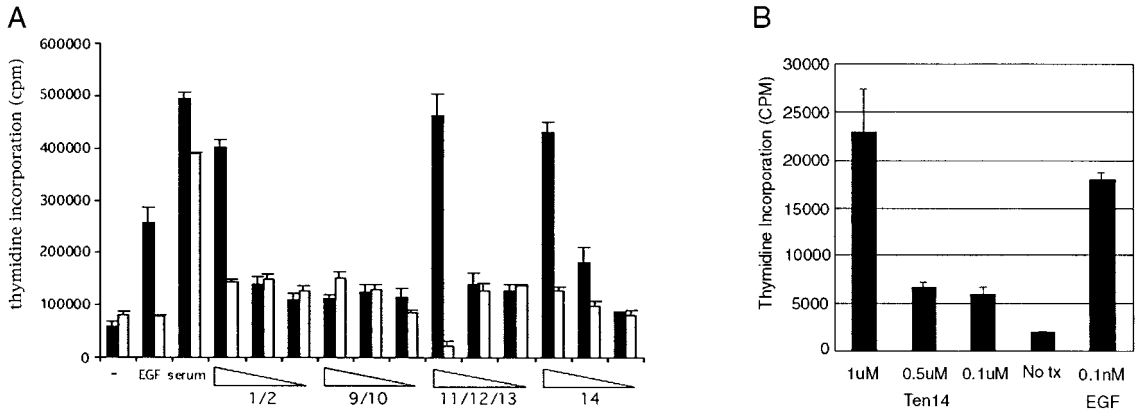


Figure 1. **Tenascin-C EGF–like repeats stimulate cell mitogenesis.** (A) WT (black) or M (open) NR6 cells were exposed to the EGF-like repeat proteins, and ^{3}H-thymidine incorporation was assessed. EGF (1 nM) and serum (1%) were used as positive controls (for WT and M NR6 cells, respectively). The EGF-like repeat proteins were used at the following concentrations: left bars are 2 uM for 1/2; 1 uM for 9/10; 4 uM for 11/12/13; and 3 uM for 14, and at 10 and 1% of that level for each concentration (middle and right bars). (B) Mitogenesis assay performed in serum-free media on WT NR6 cells with decreasing concentrations of Ten14 as described. No tx designates cells not exposed to ligand. Values are the mean ±SD (performed in triplicate) for one experiment representative of three experiments.

genic response in WT NR6 cells at concentrations of ~0.01 kd (Reddy et al., 1996a). Thus, the activation pattern for EGFR by the tenascin EGF–like repeats in soluble form is similar to that for an EGFR ligand at a concentration significantly below solution kd but significantly above the threshold required to stimulate a mitogenic response.

Because the degree of EGFR phosphorylation is not a sensitive indicator of the degree of mitogenic response, we also determined whether downstream signaling is initiated. A crucial pathway for mitogenesis and motility signaled by EGFR is the one leading to MAP kinase activation. Both ERK MAP kinases were dually phosphorylated in response to the tenascin repeats 1/2, 11/12/13, and 14, but not 9/10 (Fig. 2 B). Again, this was noted only in WT NR6, not M NR6 cells. That the M NR6 cells were competent was demonstrated by exposure to serum inducing both mitogenesis and ERK/ MAP kinase phosphorylation.

This pattern of relatively small phosphorylation of EGFR but robust activation of ERK MAP kinases is not unexpected if one considers the highly nonlinear effects that result from differential rates of signal activation and attenuation of molecules activated downstream from EGFR (Bhalla and Iyengar, 1999). Such nonlinearities can result, for example, in persistent activation of MAP kinase or PKC after the EGF signal is withdrawn and maximal pathway stimulation over a wide range of concentrations of signaling pathway components (Bhalla and Iyengar, 1999). The EGFR is subject to multiple signal attentuation mechanisms, including rapid dephosphorylation and internalization (Welsh et al., 1991; Countaway et al., 1992; Hernandez-Sotomayor et al., 1993), but at the same time is associated with a prolonged activation or slower deactivation of downstream signals, such as persistence of grb2–SOS interactions (Waters et al., 1996). The observed EGFR activation pattern would be expected for either a ligand at concentrations significantly below kd or a low affinity ligand with a high off-rate. This was tested with both a high affinity ligand (EGF, kd ~2 nM) and a lower affinity ligand (Y13G-EGF, kd ~100 nM; 41; Fig. 3 B). As observed, EGFR autophosphorylation was barely demonstrable at 0.1 kd for both these ligands. Notably, though, dually phosphorylated ERK MAP kinase could be detected at even lower concentrations: 0.01 kd for both ligands, and both ligands stimulated maximal MAP kinase phosphorylation at 0.01 kd. It was at approximately this level of fractional kd that Ten14 induced dually phosphorylated ERK MAP kinase (Fig. 3 C).

These data suggest that EGFR phosphorylation would be enhanced by limiting attenuation or increasing ligand accessibility. We used sodium vanadate to block receptor dephosphorylation, as this is the most rapid attenuation event (Hernandez-Sotomayor et al., 1993; Fig. 3 D). Treatment with this generalized tyrosine phosphatase inhibitor increased EGFR phosphotyrosine content after exposure to Ten14; similar vanadate-increased EGFR phosphorylation was noted in response to low levels of EGF (0.01 nM), demonstrating fidelity of the assay. To increase signaling persistence and/or ligand accessibility, we tethered EGFR ligands via the NH_2-termini to ~1-um diameter latex beads using 20 nm polyethylene oxide (PEO) flexible spacer chains to ensure ligand accessibility. This represents an initial attempt to present low affinity ligands in a context that mirrors ligands constrained within the extracellular matrix (Kuhl and Griffith-Cima, 1996). When the tenascin 14 repeat was covalently tethered to these beads, a significantly higher level of tyrosyl-phosphorylation was observed over that obtained with soluble, monomeric ten14 (Fig. 3 E). It was noted that EGFR phosphorylation increased with time exposed to the tenascin-tethered beads; however, it remains to be determined whether this is due to slow diffusion and settling of beads or reflects a situation akin to eph receptor activation (Davis et al., 1994). These data support the finding that select tenascin EGF–like repeats directly activate the EGF receptor from an insoluble presentation mode. Furthermore, the initial findings with these insoluble ligand complexes strongly suggest that manner of ligand presentation alters the balance between signaling and attenuation.

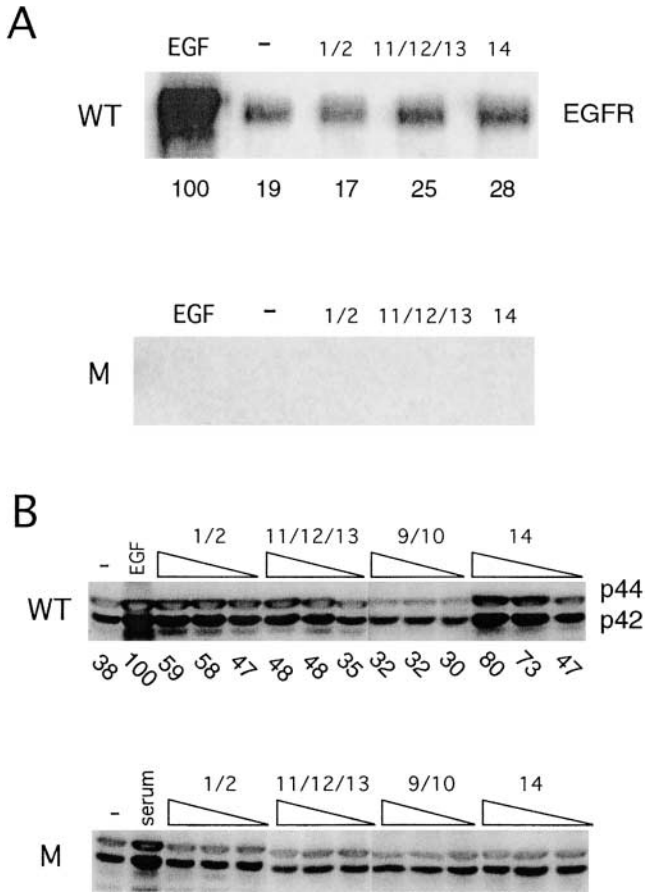


Figure 2. **Tenascin EGF–like repeats activate the EGFR kinase cascade.** WT (top) and M (bottom) NR6 cells were treated with EGF-like repeat proteins and activation of EGFR signaling was assessed. (A) EGFR autophosphorylation was determined by antiphosphotyrosine immunoblotting of immunoprecipitated EGFR after treatment with EGF or EGF-like repeats 1/2 (5 uM), 11/12/13 (1 uM), or 14 (6 uM). Immunoblotting with an antibody to EGFR demonstrated equal loading (data not shown). (B) ERK MAP kinase activation was assessed by immunoblotting for dually phosphorylated p44/p42 ERK, indicative of activated ERK. The cells were treated with various concentrations of the EGF-like repeats (5 uM for 1/2; 2 uM for 9/10; 1 uM for 11/12/13; and 6 uM for 14) and at 50 and 10% of those levels. EGF and serum were used as positive controls (for WT and M NR6 cells, respectively). Numbers below a lane represent relative values of intensity for pEGFR or p42 in each lane for that experiment as determined by densitometry. In both panels, an experiment representative of at least three determinations are shown.

The MAP kinase signaling pathway is activated by the EGF-like repeats of tenascin-C through their direct activation of EGFR

That EGFR kinase activity is required for downstream cell responses was corroborated directly by inhibiting EGFR using the pharmacological agent PD153035. This selective inhibitor of EGFR kinase blocked ERK MAP kinase activation by the tenascin EGF–like repeats (Fig. 4 A).

The foregoing results may be due to the tenascin repeats interacting with an unknown receptor but transmitting signal through an intact EGFR kinase, as has been reported recently for G protein–linked receptors (Daub et al., 1996), integrins (Li et al., 1999), and growth hormone receptor (Yamauchi et al., 1997). To probe this unlikely possibility, we blocked the external binding site of known EGFR ligands using a nonactivating antibody (clone 528; Sunada et al., 1986). The extracellular domain antibody inhibited the induced MAP kinase activation (Fig. 4 B), indicating that activation of the EGFR by tenascin-C EGF–like repeat occurs through the ligand binding, extracellular domain of the EGFR. However, it is still possible that the signals occur by a receptor cascade in which the unknown receptor causes release of membrane-anchored EGFR ligands that then act in autocrine fashion, as has been shown for G protein–coupled receptors (Prenzel et al., 1999). This is unlikely, as NR6 cells are not known to produce significant levels of EGFR ligands. However, we challenged B82 cells that have been shown not to express any of the known EGFR ligands (Oehrman et al., 1998) with the tenascin 14 fragment (Fig. 4 C). The tenascin 14 fragment activated ERK MAP kinase similarly to what had been noted in WT NR6 cells.

Tenascin EGF–like repeats directly bind to EGFR

Final proof that these EGF-like repeats act as novel direct EGFR ligands, however, requires a visualization of EGFR-dependent binding and/or a demonstration of an interaction with EGFR. In direct binding assays, we were unable to detect specific binding to the EGFR by the EGF-like repeat proteins at micromolar concentration (data not shown). This indicates that the ligand is quite low affinity ($\geq$10 uM) when compared with prototypical growth factor ligands for EGFR (in the low nanomolar range). This proposed low solution affinity of the tenascin 14 repeats appears to be commensurate with the solution affinities for integrin ligands, which are in the micromolar range for fibronectin (Akiyama and Yamada, 1985) and the millimolar range for linear arginine–glycine–aspartic acid peptides (Pierschbacher and Ruoslahti, 1987). Since specific binding to these integrin ligands can be readily detected by presenting the ligands from the solid phase (i.e., bound to beads or the substrate; Pierschbacher and Ruoslahti, 1984) in a relatively normal physiological manner, we reasoned that specific binding to the tenascin 14 repeats might also be detected by presenting the repeats in a method that resembles their presentation in ECM. We generated $\sim$1-um diameter beads that presented a high surface density of ligand (either tenascin 14 or EGF). Beads presenting EGF or the tenascin 14 fragment exhibited specific adhesion to WT NR6 cells compared with control beads (Fig. 5). That this occurred via EGFR is demonstrated by blocking of binding by anti-EGFR antibodies (number of bound beads were reduced by $>$90% in each of three independent experiments). There was negligible binding to M721 NR6 cells, which are devoid of EGFR.

Direct interaction of the EGF-like repeats with EGFR should be enhanced by increasing the valency of the ligand, with even dimerization sufficient to achieve a log-greater avidity (Mammen et al., 1998). Ligands were predimerized with an antibody to the poly-His tag at the NH_2 terminus of the expressed repeats. The binding of these ligands to WT NR6 cells was visualized by indirect immunofluorescence (Fig. 6). As a positive control, murine EGF (mEGF-His6) was cloned and purified in a similar manner as the tenascin EGF–like repeats. Ten14 dimers bound at a level significantly greater than antibody alone. Most importantly, this

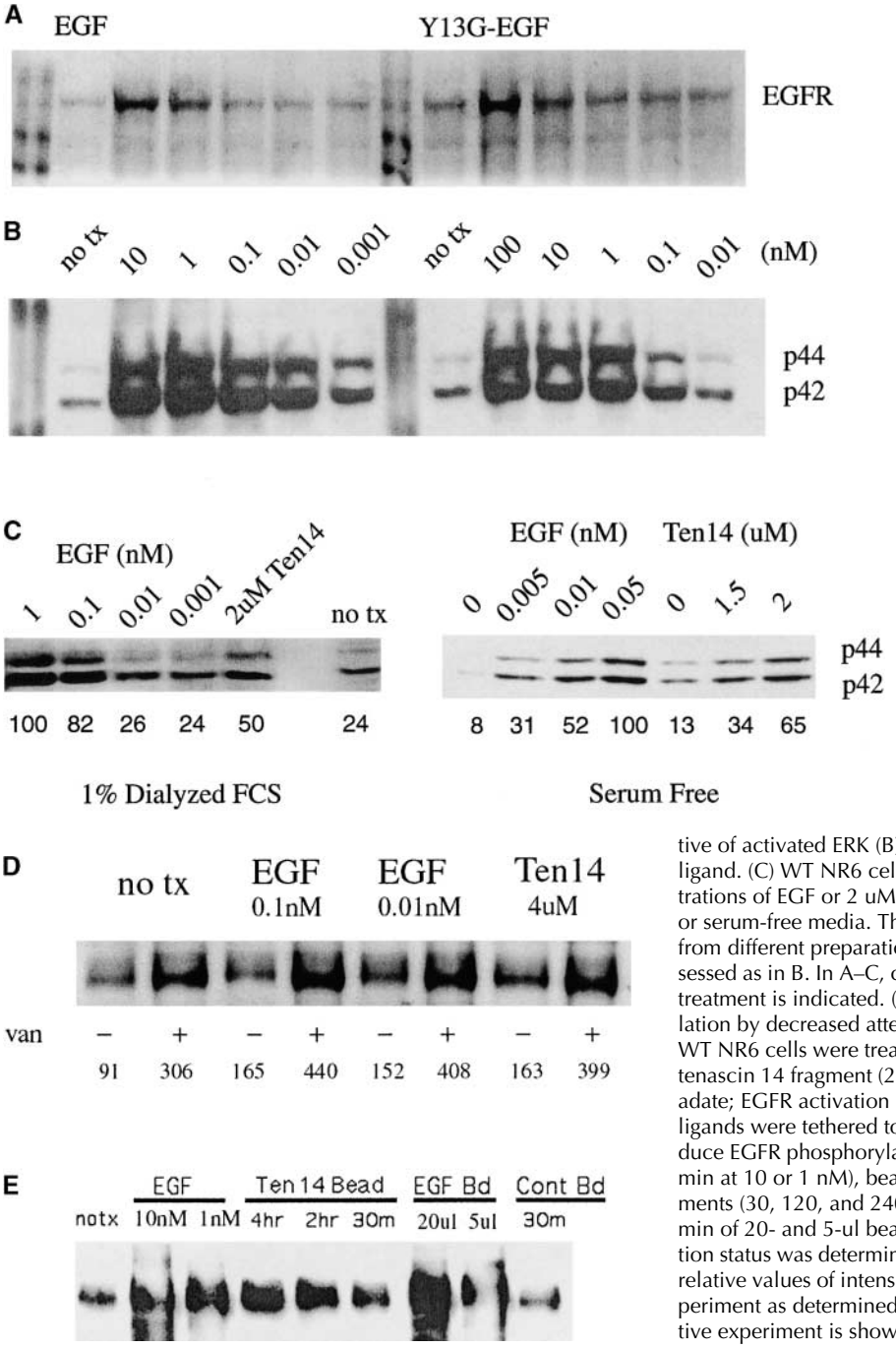


Figure 3. **Differential detection of activation of the EGFR kinase cascade.** (A and B) WT NR6 cells were treated for 5 min with decreasing concentrations of a high affinity (EGF, kd ~2 nM; left) or lower affinity (Y13G-EGF, kd ~100 nM; right; Reddy et al., 1996a) EGFR ligand. Activation status of EGFR was determined by immunoblotting with the PY20 antiphosphotyrosine antibody of 175 kD (A), and ERK MAP kinase activation was assessed by immunoblotting for dually phosphorylated p44/p42 ERK, indicative of activated ERK (B). no tx designates cells not exposed to ligand. (C) WT NR6 cells were treated as in B with various concentrations of EGF or 2 uM tenascin 14 fragment in quiescence media or serum-free media. The two different concentrations of Ten14 are from different preparations. ERK MAP kinase activation was assessed as in B. In A–C, concentration in nM of EGFR ligand used for treatment is indicated. (D and E) Augmentation of EGFR phosphorylation by decreased attenuation mechanisms in WT NR6 cells. (D) WT NR6 cells were treated for 30 min with EGF (0.1 or 0.01 nM) or tenascin 14 fragment (2 uM) in the presence of 0.1 mM sodium vanadate; EGFR activation status was determined as in A. (E) EGFR ligands were tethered to PEO latexes and tested for the ability to induce EGFR phosphorylation. WT NR6 cells were exposed to EGF (5 min at 10 or 1 nM), bead complexes containing tenascin 14 fragments (30, 120, and 240 min), bead complexes containing EGF (30 min of 20- and 5-ul beads), or control beads (30 min); EGFR activation status was determined as in A. Numbers below a lane represent relative values of intensity for pEGFR or p42 in each lane for that experiment as determined by densitometry. In all panels a representative experiment is shown of at least three determinations.

binding, and that of mEGF-His6, was competed by 100 nM EGF that did not present the poly-His tag. Interestingly, Ten14 that was not predimerized also demonstrated statistically significant cell association, though at a slightly lower level than the predimerized Ten14. These data, including the bead-binding study, strongly support the model of low affinity ligands interacting with the EGFR.

The final demonstration would be to biochemically detect an association between EGFR and Ten14. To confirm the immunofluorescence of Ten14 binding to EGFR, we cross-linked the ligands to their receptors and examined immunoprecipitates of the poly-His tags. Upon poly-His immunoprecipitation, we detected EGFR in cells dithiobis(succinimidyl propionate) cross-linked in the presence of either mEGF-His6 or Ten14 (Fig. 7 A). This interaction was not noted in cells competitively treated with 100 nM unlabeled EGF. Diluent (notx) or just secondary antibody (IgG) alone did not identify any cell surface receptor. Furthermore, we assessed specificity by probing for another cell surface receptor, the insulin receptor (Fig. 7 B); we could not detect any interaction between Ten14 or mEGF-His6 and this receptor, further establishing the fact that binding is specific for EGFR. That Ten14 coprecipitated at least similar levels of EGFR, as did mEGF-His6, is not unexpected. The extended period of cross-linking would minimize the effects of the rapid off-rate predicted for Ten14 and thus "drive" the binding towards completion. The slight retardation of migration for EGFR noted is also not unexpected for

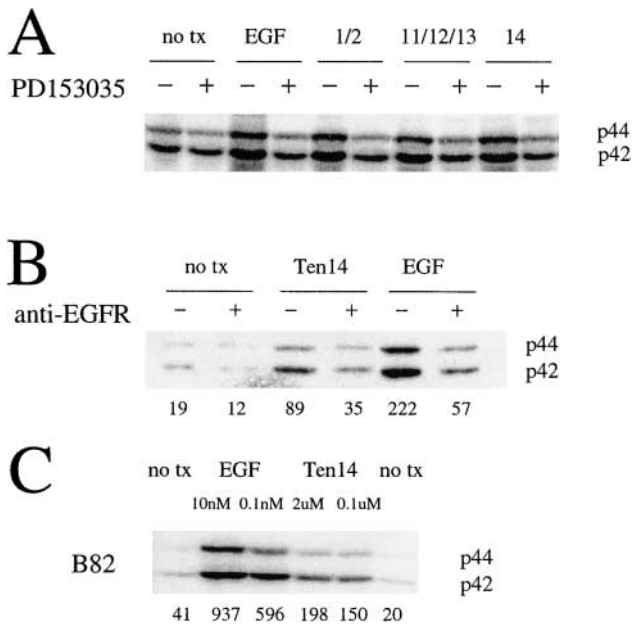


Figure 4. **Inhibition of EGFR activation prevents signaling from EGF-like repeats.** (A) WT NR6 cells were treated with the tenascin EGF–like repeats at concentrations that activate ERK MAP kinase (5 uM for 1/2; 1 uM for 11/12/13; and 6 uM for 14). The cells were treated with the EGF-like repeat proteins in the absence (−) or presence (+) of the EGFR-specific pharmacologic inhibitor PD153035. no tx represents no ligand. (B) WT NR6 cells were treated with Ten14 (1 uM) or EGF (0.01 nM) in the presence (+) or absence (−) of an antibody specific for the extracellular domain of EGFR (Clone 528; Calbiochem) under serum-free conditions. (C) B82 cells expressing WT EGFR were challenged with tenascin 14 repeats (2 uM) under serum-free conditions. Numbers below a lane represent relative values of intensity for p42 in each lane for that experiment as determined by densitometry. Shown are one of three experiments.

chemically cross-linked proteins. In summation, these data demonstrate that Ten14 directly binds to EGFR.

Discussion

These data indicate that select EGF-like repeats may act as low affinity ligands of the EGFR. We could detect binding to EGFR upon tethering tenascin fragments to a bead to present a multivalent ligand in a manner similar to a matrix-embedded situation, a situation which is predicted of low affinity ligands and has been observed for integrin ligands (Juliano et al., 1993; Maheshwari et al., 2000), and by increasing the valency by dimerization with antibody. Most conclusively, we could cross-link an EGF-like repeat to EGFR. It is not surprising that these repeats are of low affinity, since they lack the second loop of the EGF structure that is required for high affinity interaction with the receptor (Montelione et al., 1987; Engler et al., 1988; Tadaki and Niyogi, 1993). Still, these tenascin EGF–like repeats may represent a new class of matrix-encoded ligands for growth factor receptors ("matrikines"), further supporting the opinion that "there is little or no justification for drawing a distinction between adhesion receptors and receptors for soluble ligands...indeed, many "soluble" growth factors often do not function as truly soluble molecules" (Hynes, 1999).

It has been suggested previously that EGF-like repeats potentiate signaling from growth factor receptors through the activation of integrins and other signaling transducers (Jones et al., 1997). The entire matrix protein tenascin-C presents numerous interactive and signaling elements. However, we do not feel that this indirect action explains the signaling from the EGF-like repeats. First, these repeats activated biochemical and biological responses in the absence of soluble factors (i.e., serum-free conditions) dependent on binding to a signaling competent EGFR (Figs. 1–4). These data do not exclude the possibility of signaling through release of membrane-associated EGFR ligands (Daub et al., 1996; Prenzel et al., 1999). This scenario is countered by the second set of data, those demonstrating direct interactions between Ten14 and EGFR (Figs. 5–7). We could demonstrate EGFR-specific bead binding and Ten14 binding and cross-linking. These interactions were prevented by anti-EGFR antibodies and competed by unlabeled EGF, demonstrating that they occurred through the ligand binding site of EGFR. In short, we have provided definitive evidence for direct binding of EGFR by EGF-like repeats.

The physiological role of such a low affinity ligand remains an open question. One of the main attenuation mechanisms necessary to prevent excessive signaling is EGFR internalization and subsequent degradation of receptor and/or ligand (Welsh et al., 1991; Reddy et al., 1996b). For a ligand that is embedded in the extracellular matrix, such internalization would be physically limited and constantly represent itself even after dissociation due to its physical proximity to the cell surface. A matrix-embedded ligand with high affinity would lead to continuous, strong signaling through EGFR; this has been shown to lead to cellular transformation (DiFiore et al., 1987; Wells et al., 1990). In contrast, a ligand with low affinity (fast off rate; Ebner and Derynck, 1991) would result in ligand decoupling from receptor, allowing dephosphorylation and other mechanisms (Welsh et al., 1991; Countaway et al., 1992; Hernandez-Sotomayor et al., 1993) to function more efficiently at attenuating signaling.

Tenascin-C is an excellent candidate to present such activators of a receptor that can stimulate both cell proliferation and migration. Tenascin-C is expressed, by and large, only during periods of organogenesis and remodeling, such as the fetal/neonatal growth period and during wound repair, and is expressed as a hexamer allowing for physiological presentation of multiple potential matricrine ligands (Schalkwijk et al., 1991; Whitby et al., 1991; Erickson, 1993; Chiquet-Ehrismann, 1995). During these events EGFR signaling is required both for proliferation and migration of the cells (Ashcroft et al., 1995; Miettinen et al., 1995; Sibilia and Wagner, 1995; Threadgill et al., 1995; Xie et al., 1998; Kim et al., 1999). In support of such a model, we have found that NR6 fibroblasts expressing motility competent WT EGFR (8) present greater transmigration of the human extracellular matrix Amgel than NR6 cells expressing the motility-deficient c'973 EGFR (26.8 ± 0.2% vs. 9.6 ± 0.3% of the invasiveness of highly invasive HT1080 fibrosarcoma cells; $P < 0.01$). This result is similar to transmigration actuated by EGFR triggered by ligand derived either from autocrine signaling (Xie et al., 1995) or the extracellular milieu (Chakrabarty et al., 1995; Kassis et al., 1999). This is intriguing as

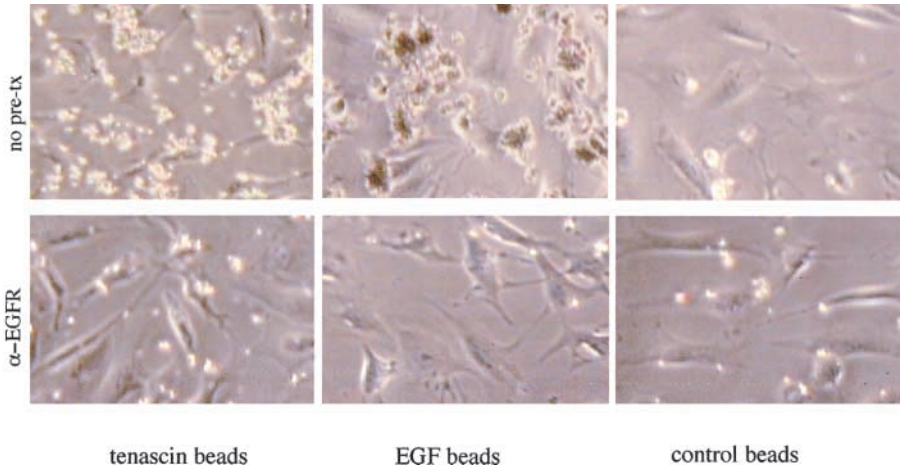
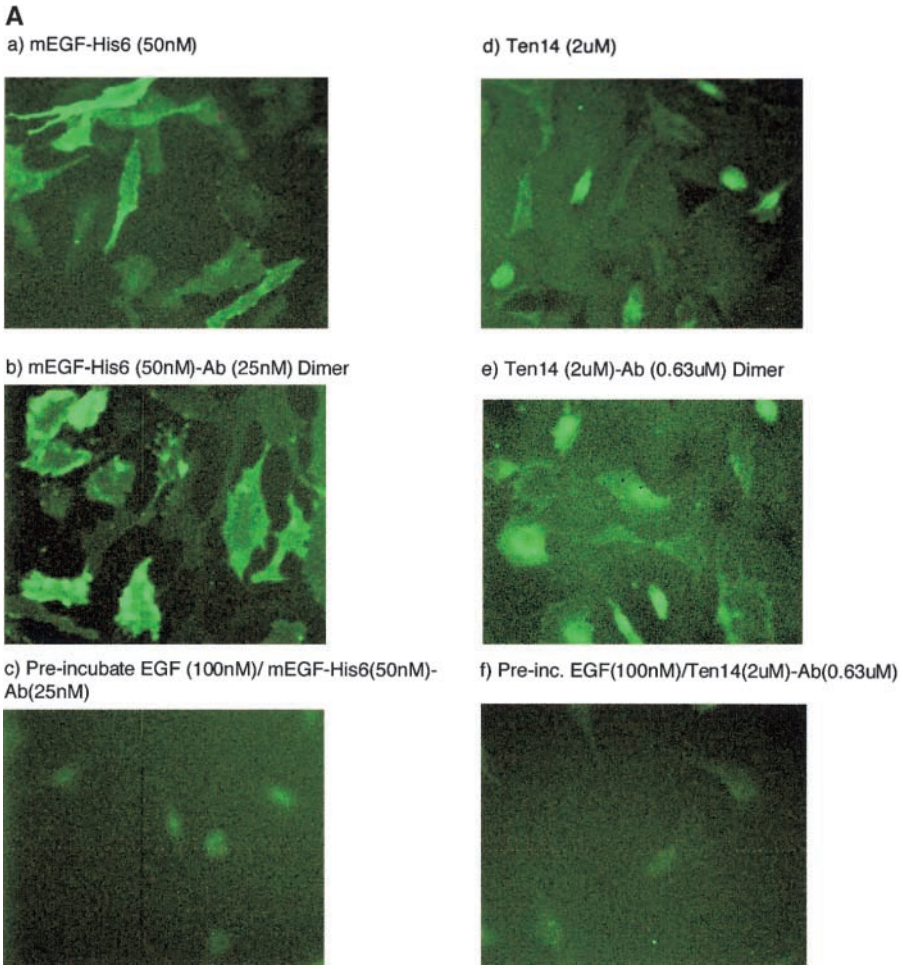


Figure 5. **Beads presenting tethered tenascin 14 fragments bind to EGFR.** WT NR6 cells were treated for 4 h at 37°C with beads tethered with either tenascin 14 or EGF or control beads. Cells were washed three times with iced PBS and visualized by phase-contrast microscopy (top). In parallel, cells were treated with 5 ug/ml anti-EGFR antibody (clone 528; Sunada et al., 1986; Turner et al., 1996) for 15 min before addition of beads and continuing throughout the 4 h (bottom). Beads appear as bright, refractile, and translucent spheres mainly above the plane of the cells. Shown is one of three experiments.



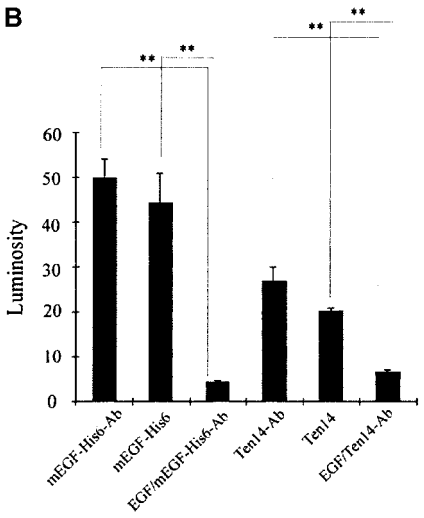


Figure 6. **Tenascin 14 binds to EGFR-expressing WT NR6 cells.** (A) WT NR6 cells were quiesced for 24 h and exposed to various concentrations of monomeric or dimerized ligand to visualize binding to EGFR. Ten14 and mEGF-His6 expressed from the same vector (pRSETA; Invitrogen) as Ten14 were incubated overnight for dimerization with monoclonal anti-HisG (Invitrogen) antibody that recognized the NH_2-terminal poly His epitope of mEGF-His6 and Ten14. This was at concentrations of 50 nM for mEGF-His6 and 25 nM for monoclonal anti-HisG antibody and 2 uM Ten14 with 0.63 uM of the antibody (Ab) to increase affinity to receptor. Ligands were incubated for 10 min at room temperature before fixation. 1:500 of goat anti–mouse antibody conjugated to Oregon green was used as secondary antibody before visualizing by fluorescence microscopy and captured at a constant exposure by a SPOT II CCD camera. a, mEGF-His6 (50 nM); b, mEGF-His6 (50 nM) dimerized with primary antibody (25 nM); c, cells were preincubated with EGF (100 nM) for 5 min to compete for EGFR with mEGF-His6 (50 nM) dimerized with antibody (25 nM); d, Ten14 (2 uM); e, Ten14 (2 uM) dimerized with primary antibody(0.63 uM); f, cells preincubated with EGF (100 nM) for 5 min with Ten14 (2 uM) preincubated with 0.63 uM antibody subsequently added. (B) Each cell in four randomly selected fields were outlined and measured for luminosity as compared with background. The data are the mean ±SE of an average of >25 cells per experimental condition. Statistical analyses were performed via Student's *t* test. Double asterisk represents $P < 0.01$. Shown is one representative of two sets of experiments.

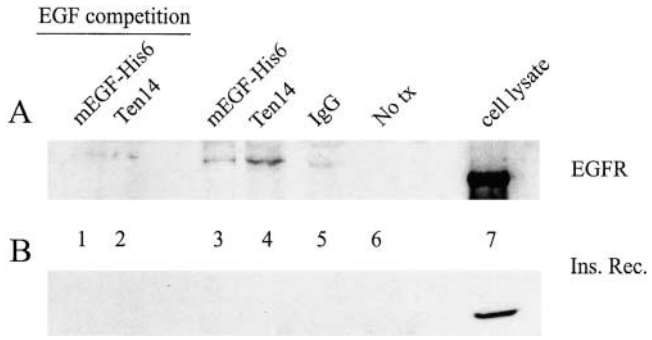


Figure 7. **Tenascin 14 can be cross-linked to EGFR.** Ten14 (2 uM) and mEGF (mEGF-His6; 10 nM) were bound and chemically cross-linked to quiesced WT NR6 fibroblasts and immunoprecipitated from ensuing lysates with anti-HisG. Presence of EGFR (A) or insulin receptor β chain was assessed by immunoblotting with respective antibodies. In lanes 1 and 2, cells were preincubated with 100 nM EGF for 5 min and throughout cross-linking as a competitive ligand. In lane 5, cells were exposed to and cross-linked with antibody alone and lysate was immunoprecipitated. From left to right: 1, EGF/mEGF-His6 (pretreatment with EGF and addition of mEGF-His6 ligand); 2, EGF/Ten14 (pretreatment with EGF and addition of Ten14 ligand); 3, mEGF-His6 (10 nM); 4, Ten14 (2 uM); 5, IgG (anti-HisG antibody); 6, No tx (no treatment with ligand); and 7, cell lysate (WT NR6 lysate). Shown is a representative of two experiments.

NR6 cells do not produce known EGFR ligands, and Amgel, derived from human amniotic membranes (Siegal et al., 1993), does not contain detectable levels of EGF, TGF-α, or other soluble EGFR ligands, but does contain appreciable levels of tenascin (75 mg/ml out of ~1,300 mg/ml proteinaceous material). Thus, low affinity ligands encrypted within matrix components might represent a new mode of modulation of cellular responses by matrix acting directly through growth factor receptors.

Materials and methods

Cell lines and plasmids

The establishment and maintenance of the WT and M NR6 cell lines have been described previously (Wells et al., 1990; Chen et al., 1994). In brief, cells were grown in MEM with 7.5% fetal calf serum and 350 ug/ml G418. Cells were quiesced in MEM containing 1% dialyzed fetal calf serum. Failure to adequately quiesce the cells results in higher background phosphorylation of EGFR and ERK even in the absence of exogenous added EGFR ligand. The percentage of dialyzed serum and length of time of the quiescence before testing must be empirically determined for each lot of dialyzed serum. For the NR6 cells and our lots of serum, 24 h of quiescence was sufficient. Tenascin-C cDNA was generated from human placental RNA by reverse transcriptase PCR using tenascin-C–specific primers (cgcggatccggccccaactgctctgagc and ccggaattcagacacctctgagcagtc), and ligated into pTrc-His-A (Invitrogen) to yield plasmid pTrc-ten. DNAs coding for specific EGF-like repeat regions within tenascin-C were generated from the pTrc-ten template by PCR and ligated into pRSET-A (Invitrogen) to yield plasmids pTen-1/2, -9/10, -11/12/13, and -14 with the numbers corresponding to the order with which each repeat occurs within tenascin C. Sequences of primer pairs used in the PCR were cgcggatccggccccaactgctctgagc and ccggaattcgatttcacggctgcagtc for pTen-1/2; cgcggatccagccagctacggtgc and ccggaattcttggcgatcccggcag for pTen-9/10; cgcggatcccgggatcgccaatgc and ccggaattcggagtgctggccacag for pTen-11/12/13; and cgcggatccggccagcactcctgc and ccggaattcagacacctctgagcagtc for pTen-14. mEGF-His6 was similarly cloned to serve as a control. These clonings yielded the EGF-like repeats and mEGF preceded by poly-His.

Expression and purification of EGF-like repeat proteins

Midlog phase cultures of *Escherichia coli* strain BL21/DE3/pLys-S (Stratagene) transformed with the individual expression plasmids were induced for recombinant protein expression with 1 mM isopropyl-b-D-thiogalactopyranoside for 4 h at 37°C. Bacteria were harvested by centrifugation for 10 min at 5,000 *g* at 4°C, and bacterial lysates were prepared by extraction with 0.02 culture volumes of B-PER detergent (Pierce Chemical Co.). Recombinant proteins were purified from bacterial lysates by nickel-agarose chromatography with imidazole elution. Purified protein was dialyzed against PBS, 0.25 mM 2-mercaptoethanol for 24 h at room temperature.

Mitogenesis assay

Cells were quiesced for 24 h under normal growth conditions in starvation medium (serum-free growth medium supplemented with 1% dialyzed fetal calf serum). The ligand-induced ^{3}H-thymidine incorporation assay has been described previously (Chen et al., 1996). In brief, cells were exposed to EGF (1 nM), serum (1%), or various concentrations of EGF-like repeat proteins for 24 h. ^{3}H-thymidine was added to the cells for the last 8 h to determine stimulation of proliferation.

Phosphorylation assays

For assaying EGFR activation, quiesced cells were treated with ligand for 5 min in quiescence medium (for the experiments described in Fig. 3 D only, the medium was supplemented with 0.1 mM sodium vanadate during this time period and treated for 30 min). When indicated in the figure legends, after cells were quiesced the experiment was performed under serum-free conditions; this further reduces background phosphorylation of ERK. Detergent lysates were immunoprecipitated at 4°C with anti-EGFR antibody (Ab-1; Oncogene Research Products) bound to protein A–conjugated agarose (GIBCO BRL). Immunoprecipitated EGFR was analyzed for tyrosine phosphorylation by immunoblotting using antiphopshotyrosine antibody (PY20; Transduction Laboratories). For assessing MAP kinase activation, quiesced cells were treated with ligand for 5 min in the presence or absence of anti-EGFR Ab-1 (4 ug/ml; Calbiochem) or PD153035 (1 uM). Whole cell lysates were analyzed for dually-phosphorylated ERK MAP kinase by immunoblotting using antiphospho-MAP kinase antibody (New England Biolabs, Inc.). Equal loading was assured using the pan-erk antibody. Relative densitometric values were derived with the NIH Image shareware and Adobe Photoshop® software.

Tethered ligands

Tenascin 14 fragments and EGF were covalently tethered to surfaces to present the ligands in a manner analogous to physiological presentation of matrix-associated tenascin. Poly(methyl methacrylate) latex beads were synthesized by dispersion polymerization using an amphiphilic comb copolymer stabilizer, following a procedure adapted from (Banerjee et al., 2000). A comb stabilizer comprised of methylmethacrylate, polyethylene glycol methacrylate (Mn = 526 g/mol), and methoxypolyethylene glycol methacrylate (Mn = 425 g/mol) in a weight ratio of 30:10:10 was synthesized by free radical polymerization using AIBN as initiator. The hydroxy-terminated polyethylene glycol side chains were subsequently carboxylated by refluxing 16 g of comb with 10 g succinic anhydride, and 0.15 ml N-methyl imidazole in 300 ml of dichloroethane overnight at 80°C. The carboxylated product was precipitated and washed with acidified water. Polyethylene methylmethacrylate latexes were synthesized by the addition of 9 ml methylmethacrylate, 1.25 g of carboxylated comb stabilizer, 1.2 ml vinyl methacrylate cross-linking agent, and 0.50 g of ammonium persulfate initiator to 45 ml of 70:30 (vol/vol) methanol/water. The reaction proceeded at 50°C for 3 h, resulting in a highly stable dispersion of micron-sized polyethylene methylmethacrylate latex beads, each coated by comb polymers that situate and become grafted at the water/bead interface. The carboxylated latex suspension was purified by repeated centrifugation and redispersion, before peptide coupling.

The NH_2-terminal amine groups of EGF and tenascin 14 fragments were used to covalently link the peptides to ends of the PEO chains emanating from the surface of the latex beads. Beads were resuspended in dry ethanol with 20 mg/mL sulfo-NHS (Pierce Chemical Co.) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Pierce Chemical Co.) and activated at room temperature for 3 h, then centrifuged to remove supernatant and resuspended in ethanol for a total of three washes. Beads were washed a final time in 100 mM phosphate buffer, pH 7, and resuspended in 5 ug/mL mouse EGF (Collaborative Biomedical), tenascin 14 fragment, or buffer alone in 100 mM phosphate buffer, pH 7. The coupling reaction was allowed to proceed for 24 h at 4°C. Unreacted peptide was removed and residual NHS reactivity blocked by washing three times in 100 mM tris buffer, pH 7. The beads were then washed once with sterile PBS before addition to cells.

Immunofluorescence

WT NR6 cells were quiesced at 50% confluency. Mouse anti-HisG antibody (Invitrogen) was incubated 24 h at 4°C with Ten14 or mEGF-His6 for dimerization. After quiescence, ligand, either without antibody or antibody-ligand mix, was added to cells in serum-free media and incubated for 10 min at room temperature. 100 nM EGF served as a competitor and was added 5 min before the Ten14 or mEGF-His6. Cells were fixed with 3% formaldehyde for 15 min at room temperature, washed three times with PBS and incubated in 1% BSA for 30 min. Cells that were exposed to ligand alone were washed twice with PBS and incubated with mouse anti-HisG antibody (Invitrogen; 1:1000) for 30 min at 37°C. Cells were washed five times with PBS and secondary goat anti–mouse conjugated to Oregon green (Molecular Probes; 1:1,000) was added at 37°C for 30 min. Cells were once again washed three times, mounted, and viewed.

Immunoprecipitation

WT NR6 cells were quiesced at 80% confluency. Cells were washed once with PBS. 100 nM EGF served as a competitor and was added 5 min before the Ten14 or mEGF-His6. Cells were than washed with PBS and incubated with ligands Ten14 (2 uM) or mEGF-His6 (10 nM) in PBS for 5 min at room temperature. In parallel, cells were incubated with just PBS (no tx) or with monoclonal anti-HisG (0.01 uM) in PBS. Dithiobis(succinimidyl propionate) (Pierce Chemical Co.) was added to the solution and the cells were placed at 4°C for 30 min. Cells were than washed with 0.2% glycine solution in PBS twice and incubated with 0.2% glycine in PBS for 5 min at 4°C followed by a final wash with 0.2% glycine in PBS once again. Cells were lysed with RIPA lysis buffer with PMSF, aprotinin, and leupeptin as protease inhibitors. 30 ul of protein G agarose beads (GIBCO BRL) and mouse anti-HisG (Invitrogen; final concentration, 0.01 uM) was added to the lysate and incubated overnight. Beads were washed for a total of five times. Lysates were separated by SDS-PAGE with 2-mercaptoethanol (to cleave the cross-linker), transferred, and immunoblotted. The upper half of the membrane was probed with a monoclonal anti-EGFR (Zymed Laboratories; 1:500) and the bottom for polyclonal antiinsulin receptor β-subunit (Transduction Laboratories; 1:1,000).

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