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An Orphan Receptor Tyrosine Kinase Family Whose Members Serve as Nonintegrin Collagen Receptors

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Summary

Mammalian cells constantly monitor and respond to a myriad of extracellular signals, often by using cell surface receptors. Two important classes of cell surface receptors include the receptor tyrosine kinases, which recognize peptide growth factors such as insulin, and the integrins, which most often mediate binding to components of the extracellular matrix. We report that the collagens serve as ligands for the previously orphan family of discoidin domain–containing receptor-like tyrosine kinases. The unexpected realization that an extracellular matrix molecule can directly serve as a ligand for receptor tyrosine kinases provides an example of ligands shared by integrins and receptor tyrosine kinases, and this finding seems likely to change prevailing views about the mechanisms by which cells perceive and respond to the extracellular matrix.

Introduction

Mammalian cells must integrate and respond to a myriad of signals from their microenvironment. Many of these signals are sensed by receptors expressed on the surface of the responding cell. Two critical classes of cell surface receptors include those known as receptor tyrosine kinases and those classified as integrins. Receptor tyrosine kinases recognize and respond to peptide growth factors such as insulin, platelet-derived growth factor, and nerve growth factor (Ullrich and Schlessinger, 1990), while the integrins most often mediate binding and attachment to components of the extracellular matrix such as collagen, fibronectin, and vitronectin (Clark and Brugge, 1995). There is increasing evidence that receptor tyrosine kinases and integrins act in coordinated fashion to modulate cellular responses involving adhesion, spreading, locomotion, proliferation, survival, and differentiation state (Clark and Brugge, 1995).

Receptor tyrosine kinases are thus named because of the tyrosine kinase domain found in the cytoplasmic portion of these receptors (Ullrich and Schlessinger, 1990). Ligand binding to the receptor ectodomain results in activation of the tyrosine kinase domain, which in turn leads to recruitment and activation of a variety of downstream signaling molecules. A number of receptor-like tyrosine kinases have been molecularly cloned based on the homologies shared by the tyrosine kinase domains of all receptors in this class (e.g., Lai and Lemke, 1991). Although presumed to have ligands, these receptor-like proteins are termed "orphans" until their ligands are indeed identified. A variety of approaches has led to the identification of ligands for previously orphan receptors. For example, the ephrins have been identified as the ligands for the Eph family of receptors (Bartley et al., 1994; Beckmann et al., 1994; Cheng and Flanagan, 1994; Davis et al., 1994); Protein S and Gas6 have been identified as ligands for the Tyro3/Sky/rse/brt/tf and Axl/Ark/UFO receptors (Stitt et al., 1995; Varnum et al., 1995); agrin has been identified as the ligand for MuSK (Glass et al., 1996); glial-derived neurotrophic factor has been identified as the ligand for the Ret receptor (Jing et al., 1996; Treanor et al., 1996); and the angiopoietins have been identified as the ligands for the Tie receptors (Davis et al., 1996; Maisonpierre et al., 1997).

Among the few remaining orphan receptor-like tyrosine kinases are two close relatives that are distinguished by a structural domain in their extracellular portions that has not been found in other receptor tyrosine kinases but that was instead first noted in the discoidin I protein of the slime mold Dictyostelium discoideum (Poole et al., 1981) and thus termed the discoidin I domain. Discoidin I domains have more recently been noted to be homologous to the constant regions of blood coagulation factors V and VIII (Wood et al., 1984; Jenny et al., 1987) and to a neural recognition molecule termed A5, identified in Xenopus laevis (Takagi et al., 1987). The two closely related receptor-like tyrosine kinases that contain discoidin I domains have been cloned by several groups and given several different names. We will refer to these receptor-like tyrosine kinases as discoidin domain receptor 1 (DDR1) for the receptor previously termed DDR (Johnson et al., 1993), NEP (Zerlin et al., 1993), Ptk-3 (Sanchez et al., 1994), Cak (Perez et al., 1994), trkE (DiMarco et al., 1993), and MCK-10 (Alves et al., 1995), and discoidin domain receptor 2 (DDR2) for the receptor previously termed Tyro10 (Lai and Lemke, 1991; Lai and Lemke, 1994), TKT (Karn et al., 1993), and CCK-2 (Alves et al., 1995). Previous studies have found that DDR1 and DDR2 are widely but differentially expressed during development and in adulthood.

Here we report the identification of the collagens as ligands that bind and activate the DDR receptors. The unexpected realization that an extracellular matrix molecule can directly serve as a ligand for receptor tyrosine kinases provides an example of ligands shared by integrins and receptor tyrosine kinases, and this finding seems likely to change prevailing views about the mechanisms by which cells perceive and respond to the extracellular matrix in their microenvironments.

Results

Identification of Potential Sources for Ligands That Bind and Activate DDR Receptors

To identify potential source(s) of the DDR1 and DDR2 ligand(s), we first engineered plasmids that could be

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used to express the DDR1 and DDR2 ectodomains individually fused to the Fc portion of human IgG1. We had previously used similar receptor–antibody fusions (termed "Rbodies") to detect and identify ligands for several other receptor tyrosine kinases, including TrkB (to detect its ligands, BDNF and NT4), members of the Eph family (to detect their ligands, collectively referred to as the Ephrins), Tyro3/Sky/rse/brt/tf and Axl/Ark/UFO (to detect their ligands, Protein S and Gas6), MuSK (to detect its ligand, agrin), and Tie1 and Tie2 (to detect their ligands, the angiopoietins) (Davis et al., 1994, 1996; Stitt et al., 1995; Glass et al., 1996; Maisonpierre et al., 1997). These previous studies had assayed Rbodies for their direct binding to cell surfaces to identify cell-associated ligands (e.g., Davis et al., 1994), or they had used Rbodies to screen conditioned media from cell lines to identify released ligands (e.g., Stitt et al., 1995; Davis et al., 1996; Glass et al., 1996). The latter screens involved covalently coupling the Rbody to the surface of a BIAcore sensor chip (Fagerstam, 1991; Johnsson et al., 1991) and then using the BIAcore to detect binding activity in conditioned media passed over the surface of this chip, or they involved immobilizing proteins in conditioned media on nitrocellulose membrane slots followed by blotting with the Rbody to detect potential ligands.

Screening of more than 200 cell lines for cell-associated or released ligands for DDR1 and DDR2, using DDR1 and DDR2 Rbodies, was performed using the three assays described above. While none of the lines revealed binding activity in their conditioned media using the BIAcore assay, cell surface binding assays indicated that four cell lines were specifically bound by both the DDR1 and DDR2 Rbodies, albeit to differing degrees, but not by control Rbodies (Figure 1A). The four cell lines displaying potential cell-associated ligand activity for both DDR1 and DDR2 included A204 (rhabdomyosarcoma), U373MG (glioblastoma), Saos2 (osteosarcoma), and HeLa (epithelioid carcinoma) (Figure 1A). Cell surface binding assays using ^{125}I-radiolabeled DDR2 Rbody, which could then be assayed for competition by excess levels of the DDR Rbodies as compared to control Rbodies, confirmed the specificity of the binding activity for DDR1 and DDR2, and competition by both DDR1 and DDR2 Rbodies demonstrated that both DDR1 and DDR2 were binding to the same potential ligand on the surfaces of these cells (Figure 1B).

Although, as noted above, none of the cell lines screened detectably released DDR1- or DDR2-binding activity as evaluated in the BIAcore assay, the conditioned media of one cell line exhibited binding activity for both DDR1 and DDR2, as evaluated in the nitrocellulose slot-blotting assay (Figure 1C). This cell line, A204, corresponded to the line exhibiting the maximum cell-associated DDR1/DDR2-binding activity (Figure 1A), strongly suggesting that the cell-associated and released binding activities of A204 corresponded to the same putative DDR1/DDR2 ligand; consistent with this possibility, a relative preference for DDR2 binding as compared to DDR1 binding was detected for A204 in both the cell surface binding assay and the slot-blotting assay (compare Figures 1A and 1C). The ability to detect putative released ligand by slot blotting versus BIAcore screening is consistent with previous observations (unpublished data) that slot blotting may in some cases provide

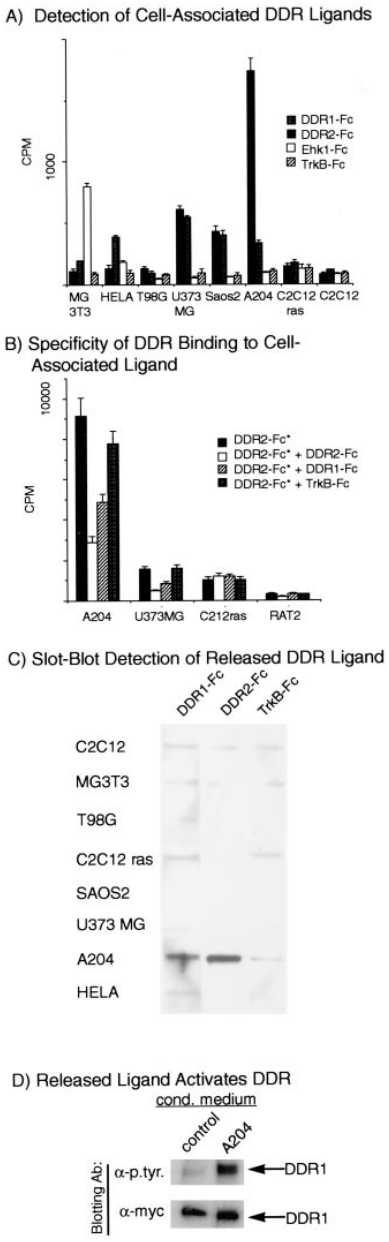

Figure 1. Detection of Cell Line Sources of Putative Ligands That Bind and Activate DDR Receptors

(A) Binding of indicated Rbodies to various cell lines identifies cell lines exhibiting cell-associated DDR ligands. Among over 200 lines screened, A204, U373, and Saos2 exhibited elevated binding to both DDR1 and DDR2 Rbodies (albeit to different relative levels) as compared to control Rbodies, while HeLa exhibited elevated binding only to DDR1; MG3T3 serves as a control cell line exhibiting elevated binding to one of the control Rbodies, Ehk1-Fc.

(B) Binding of radiolabelled DDR2-Fc to cell surfaces can be specifically competed by both DDR1 and DDR2 Rbodies (and not a control Rbody), demonstrating the specificity of binding and that both DDR receptors are binding to the same putative cell-associated ligand.

(C) Slot-blotting assay of conditioned media from over 200 cell lines demonstrates specific binding of DDR Rbodies (as compared to control Rbody) only for one cell line (A204, which exhibited highest levels of cell-associated DDR binding), providing a source of released ligand.

(D) Released ligand in the conditioned media of the A204 cell line specifically induces phosphorylation of full-length DDR1 expressed on COS cells, as compared to control conditioned media (both concentrated 50-fold).

a more sensitive assay, particularly for low-affinity ligands that may benefit from cooperative binding interactions provided by immobilized ligand being detected by a dimeric Rbody.

To provide additional support that the binding activities detected by DDR Rbody binding corresponded to bona fide ligands for the DDR receptors, we tested whether they could also activate full-length DDR receptors. For this purpose we engineered a plasmid encoding a full-length DDR1 receptor that was epitope-tagged at its caroboxy-terminus with a triple-Myc tag, then used this plasmid to express this epitope-tagged receptor in mammalian cells. These cells were subsequently challenged with either 50-fold concentrated conditioned media from control cells or from the A204 cell line, and then the introduced DDR1 receptors were immunoprecipitated using antibodies against the triple-Myc epitope and immunoblotted for phosphotyrosine levels. This analysis demonstrated that the A204 conditioned media, which contains high levels of DDR-binding activity, could induce DDR1 receptor phosphorylation (Figure 1D), providing further evidence that this DDR-binding activity corresponded to a bona fide ligand for DDR receptors.

Purification of DDR-Binding Activity from A204 Cells Identifies Collagen as a Possible Ligand

Biochemical and chromatographic analyses and separations were undertaken to characterize and purify the putative DDR ligand from the conditioned media of A204 cells; binding activity was followed using the slot-blotting assay described above. Size-exclusion chromatography revealed that DDR-binding activity behaved in a manner expected for a very large molecule: under nondenaturing conditions the activity was consistently eluting close to the excluded volume of a Pharmacia Superose 6 column (exclusion limit for globular proteins 4×10^7). Several attempts to reduce the molecular weight of the activity were undertaken. We used detergents (zwittergen 6–12, digitonin, N-dodecylmaltoside, N-octylglucoside, Nonidet P-40, Triton X-100, Tween 20, sodium deoxycholate, CHAPS), chaotropic agents (up to 6 M urea, up to 2 M guanidine hydrochloride), pH between 3 and 11, high salt concentrations, reducing agent dithiothreitol (DTT), and a combination of these various treatments; binding activity was always found in the retentates of 100 kDa filtration membranes. The activity present in A204-conditioned medium was lost in 4 M guanidine hydrochloride and, upon heating to 60°C, was resistant to the action of the nonspecific nuclease benzonase; however, it was also surprisingly resistant to pepsin and trypsin digestion, suggesting that it might correspond to a protease-resistant protein. DDR-binding activity was retained by the following chromatographic resins: cation exchange at neutral pH, anion exchange at pH 8.0, and hydrophobic interaction at 0.7 M ammonium sulfate; the activity could be eluted with specific reagents from those resins. We also found that DDR-binding activity could be precipitated out of A204 conditioned media with 20 mM calcium chloride, suggesting affinity for calcium phosphate, and then released from the precipitate using 40 mM EDTA. All our observations were consistent with a large protein factor being responsible for the binding, despite the resistance to trypsin and pepsin.

Based on the above biochemical characterizations, we developed a purification procedure to obtain a homogenous binding activity from A204-conditioned media. The purification protocol consisted of cation exchange chromatography, calcium chloride precipitation, pepsin digestion, high salt precipitation, and finally size exclusion chromatography performed in the presence of 6 M urea (Figure 2A). Silver-stained SDS polyacrylamide gel electrophoresis (SDS-PAGE) of the final product showed three bands in the region of 150 kDa (Figure

A Purification of DDR Binding Activity

	Total Protein (mg)	Total Collagen (mg)
A204 cond. media (1000ml)	337.1	13.4
↓ Cation Exchange (HiTrapS Retentate)	50.5	3.9
↓ 20mM $CaCl_2$ Precip.	ND	4.2
↓ Pepsin Digestion	ND	ND
↓ 4.5M NaCl Precip.	ND	0.35
↓ Sizing Column (Peak A) (S6 gel filtr. in urea)	0.33	0.3

B Amino Acid Composition

Amino Acid Residue	A204 Protein (mole%)	Bovine Coll. I (mole%)	Human $[\alpha_1(XI)]_2\alpha_2(V)$ (mole%)
ASX	5.4	4.7	4.7
GLX	10.2	7.0	9.4
SER	2.7	3.6	2.5
HIS	1.1	0.9	0.6
GLY	29.9	26.5	34.3
THR	1.9	1.8	2.0
ALA	6.2	11.2	5.1
ARG	4.3	5.4	4.4
TYR	0.6	0.4	0.2
VAL	2.8	2.1	2.6
MET	1.1	0.8	1.0
PHE	1.5	1.3	1.0
ILE	1.9	1.3	1.3
LEU	4.1	2.5	3.4
LYS	2.4	2.3	} 4.9
HYL	ND	ND	
HYP	11.2	15.6	} 22.2
PRO	12.6	12.6	

Figure 2. Purification of DDR-Binding Activity

(A) Scheme used to purify DDR-binding activity from A204 conditioned media; quantitation of total protein and collagen levels during the purification are provided, indicating that purification results in successive enrichment for collagen until it essentially comprises all of protein sample.

(B) Amino acid composition of protein purified from A204 conditioned media, as compared to that previously noted for purified bovine collagen I or theoretically deduced for the collagen previously shown (Kleman et al., 1992) to be produced by A204 cells (composed of 2 α1 chains from type XI and 1 α2 chain from type V); note that hydroxylysine levels were not measured, and that the theoretical amino acid composition provided does not distinguish among any hydroxylated residues.

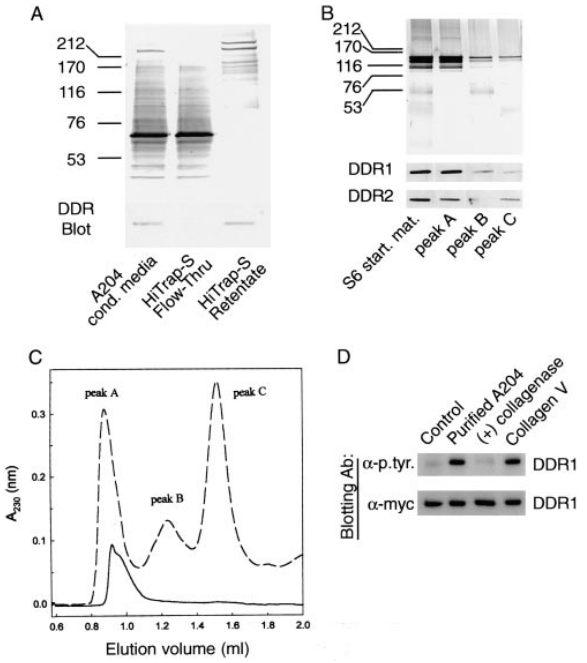

Figure 3. Gel Electrophoretic, Slot-Blot, Size Exclusion, and Phosphorylation Analysis of A204-Derived DDR Ligand during Purification Procedure

(A and B) Silver-stained gel analysis (4–20% SDS PAGE in [A], 18% SDS-PAGE in [B]), coupled with DDR slot-blot analysis, of fractions from various steps in the purification scheme outlined in Figure 2A, as indicated. In (B), note that the samples reflect material before and after Superose 6 (S6) size exclusion chromatography, with peaks as indicated in (C); most of the DDR binding is evident in peak A.

(C) Superose 6 size exclusion chromatography of material from A204 purification procedure (dashed line) as compared to purified, active collagen V (solid line) derived as described in Figure 5.

(D) DDR1 phosphorylation is induced by final purified material (peak A material, see [C]) obtained from A204 cells (second lane), abolished when this material is treated with collagenase (third lane) and is comparable to that seen with the purified collagen V (fourth lane) derived as described in Figure 5.

3B, peak A); the purified protein (after dialysis to remove the urea) was still able to induce phosphorylation of the DDR1 receptor (Figure 3D, second lane) and still active in the membrane slot-blotting assay (Figure 3B). Quantitative amino acid analysis on the isolated material revealed that almost a third of the residues were glycine and also demonstrated a high content of hydroxyproline and proline (Figure 2B), a composition highly characteristic of the triple-helical regions of the collagens.

The collagens consist of a superfamily with about 20 members that are characterized by chains composed of repeating G-X-Y sequences, in which proline is often in the X position and 4-hydroxyproline is often in the Y position, with three collagen α chains coming together to form a unique triple-helical structure (Prockop and Kivirrikko, 1995). The most abundant collagens can be split into either fibrillar-forming collagens (types I, II, III, V, and XI), in which triple helical monomers further associate to form large fibrils, or into network-forming collagens (types IV, VIII, and X), which associate to form net-like structures (Prockop and Kivirrikko, 1995). Notably, the fibrillar collagens have long uninterrupted triple-helical regions that are highly resistant to pepsin and trypsin digestion, like the binding activity we isolated from A204 cells. The A204 rhabdomyosarcoma line was previously reported (Kleman et al., 1992) to produce fibrillar heterotypic trimeric collagen molecules consisting of two α 1 chains of collagen XI and one α 2 chain of collagen V. In fact, our deduced amino acid composition was even more similar to that predicted for the helical regions of collagen types V and XI than to collagen type I, particularly with respect to the characteristically lower levels of alanine residues (Figure 2B). Furthermore, assaying for collagen during our purification procedure revealed we had achieved a dramatic enrichment for collagen (Figure 2A, last column), and the final purified material closely comigrated with pepsin-treated commercially-derived collagen type V during gel electrophoresis (data not shown) and in size-exclusion chromatography (compare dashed profile, peak A, with solid profile in Figure 3C). Finally, commercially derived human collagen type V was able to induce phosphorylation of the DDR1 receptor comparable to that of the A204-derived material (compare the second and fourth lanes in Figure 3D).

Based on all the above data, we speculated that the putative DDR ligand we had isolated from the conditioned medium of A204 cells was either collagen itself or involved a factor tightly associated with collagen that could not be displaced by the urea treatment.

Further Evidence Indicating That Collagens Are DDR Ligands

To determine whether the DDR-binding and phosphorylating activity we had been characterizing was indeed collagen, we next examined whether this activity required the production and integrity of collagen. To investigate these possibilities further, we first evaluated the sensitivity of our DDR-binding activity to microbial collagenase type VII, which can specifically degrade the triple-helical portions of collagens. Collagenase treatment of A204 and U373MG cells, which exhibited cell-associated DDR binding, effectively reduced this binding (Figure 4A); collagenase treatment did not effect background levels of control TrkB Rbody binding to these cells or that of specific B61-Fc binding to these cells (Figure 4A). Similarly, collagenase treatment (in contrast to pepsin and trypsin treatment) eliminated the DDR-binding activity (Figure 4B) as well as the DDR phosphorylating activity (Figure 3D, third lane) found in A204-conditioned medium. Furthermore, the addition of a cocktail of collagen synthesis inhibitors (*cis*-hydroxyproline [CHP] and ethyl-3,4-dihydroxybenzoate [EDHB]) to A204 cells prevented their production and release of DDR-binding activity (Figure 4B). Altogether, these data strongly suggest that the DDR-binding and phosphorylating activity we had identified in A204 and other cells corresponded to collagen itself.

To rule out further the possibility of a tightly associated factor, preparations of commercially derived human collagen type V were exploited. These preparations were noted to contain impurities and collagen fragments in addition to the intact α chains (Figure 5, lane 1), and thus we developed purification procedures that resulted in the isolation of electrophoretically pure α collagen type V (Figure 5, lane 4). The first purification step was

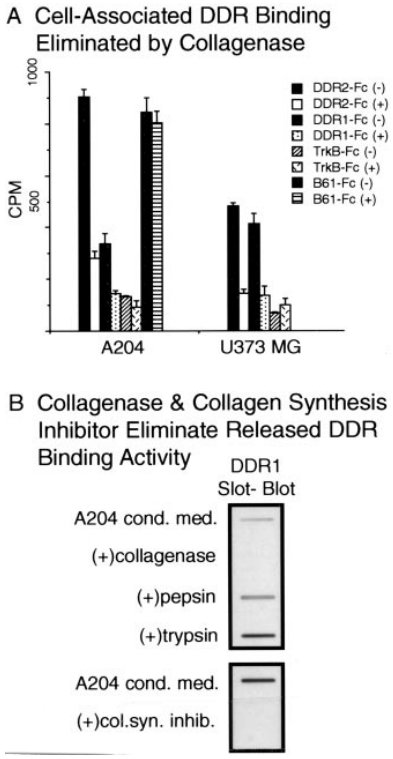

Figure 4. DDR-Binding Activity Is Abolished by Treatment with Collagenase or Collagen Synthesis Inhibitors

(A) Cell-associated DDR-binding activity exhibited by A204 and U373 cells is eliminated following treatment with collagenase. Note that U373 exhibits similar levels of binding to both DDR1 and DDR2, which are both reduced by collagenase, while A204 exhibits 2- to 3-fold more binding to DDR2 than DDR1, although binding of both DDR1 and DDR2 to A204 cells is dramatically reduced by collagenase treatment; the different relative binding of DDR1 as compared to DDR2 in the two cell lines probably reflects differences in the types of collagens synthesized by the two cell lines. The binding of B61-Fc to A204 cells serves as a control for a cell-surface binding activity that is not reduced by collagenase.

(B) DDR1 slot-blotting assay demonstrating that collagenase treatment or collagen synthesis inhibitors (CHP and EDHB) eliminate the released DDR-binding activity normally found in A204 conditioned media.

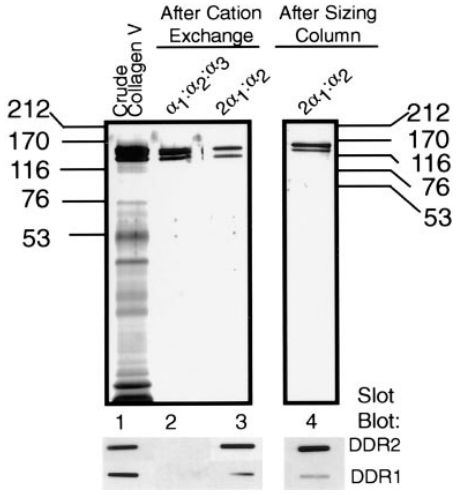

Figure 5. Gel Electrophoretic and Slot-Blot Analysis of Purification Procedure for Commercially Derived (Sigma) Human Placenta Collagen Type V

SDS-PAGE of crude collagen V (lane 1), inactive (consisting of $\alpha1{:}\alpha2{:}\alpha3$, lane 2) and active pools (consisting of $2\alpha1{:}\alpha2$, lane 3) from Fractogel EMD SO-3 cation exchange column, and active material from Superose 6 sizing column (consisting of $2\alpha1{:}\alpha2$, lane 4); activity determined by DDR slot-blotting assays depicted below gels, and also DDR phosphorylation assays (e.g., see Figure 3D, lane 4).

based on a published procedure for purification of crude collagen V (Sato et al., 1995). The crude preparation was first loaded onto a cation exchange column (Fractogel EMD SO-3) in the presence of 2 M urea. Salt gradient elution resulted in the separation of two trimeric forms of placental collagen V: $\alpha1{:}\alpha2{:}\alpha3$ trimer and $(\alpha1)_2{:}\alpha2$ trimer. We found that only the $(\alpha1)_2{:}\alpha2$ form of collagen V was active in the DDR slot-blotting assay (Figure 5, lanes 2 and 3). The active heterotrimer was subsequently run on a Pharmacia Superose 6 size exclusion chromatography column. In an attempt to dissociate collagen from any hypothetical components required for DDR binding, chromatography was performed in 6 M urea. After size exclusion chromatography, the collagen chains ($\alpha1$ and $\alpha2$ in apparent 2:1 ratio) were the only visible bands on the silver stained 18% polyacrylamide gel (Figure 5, lane 4, top). The binding (Figure 5, lane 4, bottom) and phosphorylating activity (following dialysis to remove urea; Figure 3D, fourth lane) were maintained throughout this stringent treatment, suggesting that the collagen alone is sufficient for the binding. We could not find any other protein component associated with collagen that would be required for activity. Using anion exchange chromatography (Sato et al., 1995), we separated individual α chains from the active trimer of collagen V. Slot-blotting assays showed no activity associated with individual chains, indicating that the triple-helical configuration of collagen is required for its DDR activity.

Together with the purification of collagen from A204-conditioned media as the putative DDR ligand, the resistance of this putative DDR ligand to pepsin and trypsin coupled with its sensitivity to collagenase and collagen synthesis inhibitors, as well as the inability to dissociate DDR activity away from collagen using stringent conditions, strongly indicates that collagen corresponds to the DDR ligand we have identified in A204 and other cells. Furthermore, our findings indicate that the native triple-helical configuration of collagen is required for it to serve as a DDR ligand.

Fibrillar-Type Collagens May Be Preferred DDR Ligands

We tested a variety of commercial collagens for their ability to bind and activate DDR receptors. Several of the major fibrillar collagens (types I, II, III, and V) exhibited marked binding to both DDR1 and DDR2 receptors (Figure 6A), as well as relatively strong ability to induce DDR1 receptor phosphorylation (Figure 6B); it should be noted that the same collagens from certain commercial suppliers occasionally did not reveal detectable activity (data not shown), suggesting that these collagens were inactivated for their DDR activity during purification or storage. The only nonfibrillar form of collagen tested (type IV) revealed poor binding and somewhat weaker

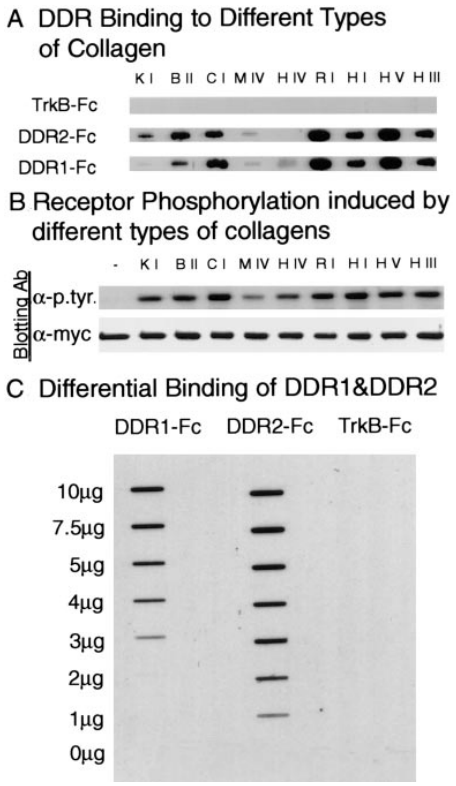

Figure 6. DDR Receptors Differentially Bound and Activated by Different Collagens

(A) DDR binding to different types of commercially-derived collagens; note weaker binding of both DDR1 and DDR2 to network-forming collagen type IV as compared to rest of collagens, which are all examples of fibril-forming collagens (K, kangaroo tail; B, bovine nasal septum; C, calf skin; H, human placenta; R, rat tail; roman numerals represent biochemical types of collagen).

(B) DDR1 phosphorylation induced by different types of collagen; note that network-forming type IV collagens consistently gave poorer phosphorylation responses.

(C) Differential slot-blot binding of DDR1 and DDR2 Rbodies to commercially-derived bovine dermal collagen I (Vitrogen). Binding was detectable for DDR2 at 1 μg loading and for DDR1 at 3 μg loading per slot.

phosphorylating activity (Figures 6A and 6B). We conclude tentatively that fibril-forming collagens may be the preferred collagen ligands for the DDR receptors, although observed differences could clearly result from differential loss of activity during the purification process for the various collagens.

We also noted slightly different preferences for DDR1 versus DDR2 binding by the various collagens. For example, bovine dermal collagen type I appears to have about a 3-fold preference for DDR2, as compared to DDR1 binding (Figure 6C). Thus, it appears possible that the DDR receptors can differentially distinguish between the various collagens.

It is also worth reexamining earlier results that different cell lines that bound the DDR Rbodies exhibited different ratios of binding to DDR1, as opposed to DDR2 (Figures 1A and 4A); for example, as noted above, A204 was preferentially bound by DDR2, while U373MG cells displayed rather equivalent binding to DDR1 and DDR2.

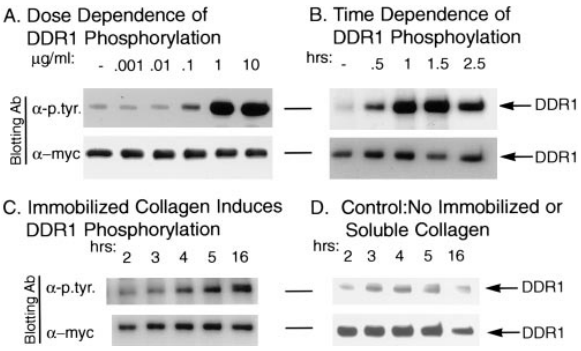

Figure 7. Dose- and Time-Dependence of DDR1 Phosphorylation Induction by Media-Added or Immobilized Collagen

(A) Dose-dependence of collagen-induced DDR1 phosphorylation; human type I collagen added to media of DDR1-expressing cells as described in Experimental Procedures.

(B) Time-dependence of collagen-induced DDR1 phosphorylation; collagen added as in (A).

(C) Immobilized human type I collagen (see Experimental Procedures) also induces DDR1 phosphorylation on cells plated on collagen, albeit with slower onset than following added collagen. (D) Control for (C), in which cells are plated on dishes that were not previously coated with collagen.

These differences seem likely to reflect differential expression by the two cell lines of the various collagen types and different modifications of the collagens that may be differentially recognized by the two DDR receptors.

Dose Dependence and Unusual Temporal Dependence of Collagen-Induced DDR Receptor Phosphorylation

We next compared the dose and temporal dependence of DDR receptor activation by collagens to those previously observed for other receptor tyrosine kinases. Maximum induction of DDR1 receptor phosphorylation appeared to require between 1 and 10 μg/ml of collagen. Surprisingly, little receptor phosphorylation was noted in the first 5 min after collagen addition, with notable phosphorylation observed only after prolonged treatment and in some cases maintained for at least 16 hr (Figure 7B and data not shown). The prolonged time for induction is in contrast to most other receptor tyrosine kinases, in which ligands induce maximum phosphorylation within minutes (e.g., Glass et al., 1996), and demonstrates that the DDR receptors may not mediate acute responses but rather tonic ones reflecting the state of collagens in the extracellular matrix.

Immobilized Collagen Also Induces DDR1 Receptor Phosphorylation

Addition of monomeric collagen solubilized in acetic acid solutions to media containing cells, as done here for the phosphorylation assays depicted thus far, results in formation of fibrillar collagen as the added collagen is brought to neutral pH. The prolonged time course noted above may also depend on time-dependent changes in the configuration of the added collagen. Such considerations emphasize the artificial nature of the experimental methods used in the receptor phosphorylation assays,

and thus an attempt was made to examine receptor phosphorylation in a more physiological manner. Thus, plates were coated with collagen to provide an immobilized ligand (albeit one enriched in monomer forms of collagen), and DDR1-expressing cells were then placed on top of this collagen. In this case, DDR1 phosphorylation was also induced with a protracted time course, while no induction was observed when DDR1-expressing cells were placed on plates that had not been coated with collagen (Figures 7C and 7D).

Discussion

Our search for ligands utilized by the previously orphan DDR1 and DDR2 receptors appears to have resulted in an unexpected convergence between receptor tyrosine kinases long known to be activated by growth factor–like peptides (Ullrich and Schlessinger, 1990) and extracellular matrix molecules, which have previously been characterized as using the integrin class of cell surface receptors (Clark and Brugge, 1995). This convergence involves the realization that collagen, whose best characterized receptors to date include the $\alpha 2\beta 1$ and $\alpha 1\beta 1$ integrins (Kuhn and Eble, 1994), can also directly bind and activate the DDR receptor tyrosine kinases. Similar findings are simultaneously being reported by Vodel et al (this issue of *Molecular Cell*). There had been considerable prior evidence, mostly from studies of platelets, that collagens might utilize nonintegrin receptors, resulting in the suggestion of a two-step model in which integrin engagement precedes binding to a second, unknown, low-affinity signal transducing receptor that initiates intracellular tyrosine phosphorylation (Kehrel, 1995; Morton et al., 1995; Asselin et al., 1997).

The realization that the collagens act as DDR ligands began with a blind search for cell line sources of an activity that could specifically bind to DDR1 and DDR2 and that could also induce DDR1 receptor phosphorylation. Once sources were identified, a purification scheme was developed to purify to homogeneity the molecule accounting for this DDR-binding and phosphorylating activity. This purification scheme depended on following the putative DDR ligand with a direct in vitro DDR1/DDR2 receptor-binding assay. The putative DDR ligand, purified to homogenity from A204 cells by using the in vitro receptor-binding assay, retained the ability to induce phosphorylation of cell-surface DDR1 receptors. This putative DDR ligand appeared to correspond to a hybrid type V/XI fibrillar collagen molecule previously shown (Kleman et al., 1992) to be produced by the A204 cells. Further evidence that collagens do indeed serve as direct DDR ligands came from the finding that all of the DDR-binding activities seen in several cell lines could be destroyed by collagenase treatment (although they were resistant to pepsin and trypsin degradation, as is characteristic for collagens) and that production of this activity could be blocked by specific collagen synthesis inhibitors. In addition, a variety of commercially-derived collagens exhibited DDR binding and phosphorylating activity. Finally, the inability to dissociate DDR activity away from collagen using stringent conditions, together with the finding that purified collagen exhibited DDR activity only when assembled into triple-helices, strongly indicates that collagen itself corresponds to the DDR ligand we have identified in A204 and other cells.

It seems likely that collagens bind to DDR receptors in a fundamentally different manner than most conventional growth factors bind to receptor tyrosine kinases. These differences may account for the inability to detect the binding of collagen passed over immobilized DDR receptors in a BIAcore assay, as well as the protracted time required to see DDR activation after collagen challenge. Both of these observations may reflect the slow association or low affinity of the collagen–DDR interaction, or perhaps time-dependent reconfigurations of the collagen or DDR receptors that must occur before stable and functional complexes can form. Alternatively, these observations would also be consistent with the above-mentioned hypothesis that collagen must initially engage integrin receptors before it can activate a second, low-affinity signaling receptor (Kehrel, 1995; Morton et al., 1995; Asselin et al., 1997).

Understanding the physiological function of the DDRs as collagen receptors may depend upon precise elucidation of the nature of the collagen forms that can activate the DDR receptors and upon elucidation of how subtle differences in collagen structure might be distinguished by the DDR receptors, which seem to bind differentially to different collagens. Such precise understanding awaits further analysis, although our current findings indicate that the native, triple-helical configuration of collagen is required for it to serve as a DDR ligand, that fibril-forming collagens may be preferred over network-forming collagens, and that immobilized monomers and possibly monomers of triple-helical collagen can bind and activate the DDR receptors. Understanding how the different collagen types and their various configurations and modifications (such as hydroxylations, glycosylations, and cross-linkings) may result in quantitative differences in DDR activation requires further investigation, but it may provide important clues into whether the DDR receptors are involved in sensing changes in collagen structure.

It is worth noting that the discoidin domains of the DDR receptors were thus named for their extensive homology to the discoidin I protein (Poole et al., 1981) of the slime mold, D. discoideum. Discoidin I is a carbohydrate-binding lectin required for normal cell adhesion, migration, and aggregation during slime mold development (Springer et al., 1984). Although our data clearly indicate that an intact and properly folded collagen peptide scaffold is required for DDR binding, it remains possible that the discoidin domains of the mammalian DDR receptors bind primarily to carbohydrate moieties presented on this scaffold. Such a possibility might reflect conservation of discoidin domain–containing proteins from slime molds to humans in terms of their carbohydrate-binding properties as well as their roles in mediating adhesive interactions.

As noted above, it is tempting to speculate that the primary role of the DDR receptors is to sense the quantity and configuration of collagens in the microenvironment and then to regulate the cellular response in terms of adhesion, migration, differentiation, survival, proliferation, and perhaps even matrix production. For example,

it has long been appreciated that an increase in the polymerization state of microenvironmental collagens can inhibit cellular migration and proliferation (Schor, 1980; Martin and Sank, 1990; Koyama et al., 1996). Because of the existing synergies and interactions already identified between integrin and receptor tyrosine kinase signaling (Clark and Brugge, 1995), it is easy to imagine that collagen might signal via its two distinct receptor classes in an integrated manner to regulate the cellular response to the surrounding microenvironment. Recent preliminary evidence suggests that DDR receptors may be dramatically regulated in situations in which collagens are thought to play important roles. For example, collagen type VI is induced in vitro during myoblast differentiation (Piccolo et al., 1995), and myoblast differentiation can be blocked in vitro and in vivo following treatment with collagen synthesis inhibitors (Saitoh et al., 1992). We have recently observed that DDR receptor mRNA levels are markedly induced during myoblast differentiation concomittantly with collagen synthesis (A. S. et al., unpublished data). Another situation in which there is an intriguing preliminary link between DDR receptor expression and alterations in collagen synthesis and function involves pathological fibrosis. Excessive fibrosis involving alterations in the quantity and quality of collagen production marks many human diseases, including hepatic cirrhosis, pulmonary fibrosis, chronic glomerulonephritis, systemic sclerosis, scarring, arterial restenosis, and postsurgical adhesions (Varga and Jimenez, 1995). In the liver, the activated mesenchymal stellate cell has been identified as the primary source of the excessive matrix and collagen synthesis that causes hepatic scarring, and the activation state of the stellate cell seems to depend on changes in collagen structure in the surrounding microenvironment (Friedman, 1993). Thus, during liver injury, the stellate cell seems to be activated by replacement of network-forming collagens by fibril-forming collagens in the subendothelial space, and the activated stellate cell then seems to perpetuate the pathological state by continuing to overproduce fibril-forming collagens while secreting metalloproteases that specifically degrade network-forming collagens (Friedman, 1993). Preliminary evidence indicates that DDR receptors are dramatically up-regulated in activated stellate cells (S. L. Friedman, personal communication), raising the intriguing possibility that collagen activation of DDR receptors plays a key role during stellate cell–driven fibrosis and that appropriate manipulation of these receptors may prove therapeutically beneficial in treating hepatic scarring and other fibrotic diseases.

Experimental Procedures

Cell Culture and Production of Media

The cell lines and culture conditions used to prepare conditioned media have been described previously (Davis et al., 1994, 1996; Stitt et al., 1995; Maisonpierre et al., 1997).

Production and Purification of Rbodies

Expression plasmids encoding the ectodomains of DDR1 and DDR2 fused to the hinge, C2, and C3 regions of human IgG1 via a bridging sequence (glycine-proline-glycine) were engineered as previously described for TrkB, Ehk1, and B61 (Davis et al., 1994). The Rbodies encoded by these plasmids were produced according to standard protocols (O'Reilly et al., 1992) in Spodoptera frugiperda Sf-21AE cells infected with baculovirus vectors bearing the respective fusion constructs. Recombinant fusion protein were then purified by protein A-Sepharose (Pharmacia) chromatography.

Screening for Rbody Binding to Cell Surfaces

Cells were plated in 6- or 24-well plates at least 48 hr before the assay. After the cells were confluent, media from the cells were removed and replaced with a 2 μg/ml solution of the Rbody in phosphate-buffered saline (PBS) with 10% bovine calf serum (BCS). One hour later the Rbody solution was removed, cells were washed three times in PBS/10% BCS; and cells were then incubated with ^{125}I-radiolabeled secondary antibody (DuPont/NEN) in PBS/10% BCS. Finally cells were washed three times in PBS/10% BCS, solubilized in 0.1 M NaOH, and bound radioactivity quantitated.

Rbody Slot-Blot Assay

Slot blots were performed on 0.45μg nitrocellulose (SandS) or nylon membranes. Membranes were prewetted with PBS (containing Ca^{2+} and Mg^{2+}) and placed on prewetted filter paper. Samples were loaded into wells and drawn through by vacuum. Blots were blocked in a solution of 5% BCS and 5% nonfat milk in TBS-T (Tris-buffered saline, 25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, [pH 7.4]) for 1 hr at room temperature or 12 hr at 4°C overnight. Blots were washed three times for five minutes each with TBS-T and then incubated at room temperature for 1 hr in 2.5 μg/ml solution of receptor-body in 2.5% BCS-2.5% NFM/TBS-T. Blots were washed three times for five minutes each with TBS-T and then incubated for 1 hr at room temperature with a 1:5000 dilution of goat anti-human IgG (Fc-sp) antibody HRPO conjugate (Caltag, #10007) in 2.5% BCS-2.5% NFM/TBS-T. Blots were washed three times for five minutes each with TBS-T and two times for five minutes each with PBS. Blots were developed using ECL detection system (Amersham) and exposed on film (Kodak Scientific Imaging Film XAR-5) for 1–2 min exposures. As a control, duplicate blots were incubated with unrelated receptor-bodies for which positive controls were available, such as TRKB-Fc detecting BDNF.

Collagenase and Collagen Inhibitor Treatments

Cells were treated with collagenase (clostridiopeptidase A, type VII, Sigma) at 200 U/ml in PBS for 1 hr at 37°C. Conditioned media was treated with collagenase, pepsin, and trypsin by incubating a 1.5 ml aliquot of media at 37°C for 5 hr with 5000 IU/ml of collagenase and then stopping the reaction by addition of EDTA to the final concentration of 10 mM; and by adjusting the pH of 1.5 ml of conditioned medium to pH 2.5 with acetic acid and incubating overnight at 4°C with 67 IU/ml of pepsin and then stopping the reaction by adjusting pH to 7.4 with NaOH; and by adjusting a 1.5 ml aliquot of conditioned medium to 1.0 M Tris (pH 8.2) and 50 mM EDTA, incubating with trypsin at 2.5 μg/ml at room temperature for 5 hr and inactivating the trypsin by addition of PMSF to 2 mM. For the collagen synthesis inhibitor studies, A204 cells were grown to confluence and then switched to defined medium containing 50 μg/ml *cis*-hydroxy-proline and 400 μM ethyl 3,4-dihydroxybenzoate and kept in tissue culture for up to 3 days.

Purification of DDR-Binding Activity from A204 Condition Media

One liter of A204 serum-free conditioned media was loaded at neutral pH (7.4) onto a 10 ml HiTrap S cation exchange column, and the activity was eluted with 200 ml of a 0.15–1.0 M NaCl gradient. Positive fractions were identified using the DDR Rbody slot-blotting assay. Activity was precipitated from the pool of active fractions with 20 mM calcium chloride. The precipitate was taken up in 40 mM EDTA and dialyzed overnight against 0.5 M acetic acid containing 0.15 M NaCl. Pepsin was added to the solution to the final concentration of 50 IU/ml (20 μg/ml), and the sample was incubated at 4°C for 24 hr. Sodium chloride concentration in the sample was adjusted to 1.2 M. The resulting precipitate was recovered after high-speed centrifugation and dissolved in 40 mM Tris-HCl buffer (pH 8), containing 1 M NaCl. Proteins were again precipitated with 4.5 M NaCl. The precipitate was redissolved in 40 mM Tris HCl containing 1 M NaCl and injected onto 3.2 × 30 Pharmacia Superose

6 PC column equilibrated at 4°C in 40 mM Tris HCl buffer containing 150 mM NaCl and 6 M urea. The column was run at 0.04 ml/min using Pharmacia Smart System. The effluent was monitored at a wavelength of 230 nm. The purification process afforded about 0.3 mg of total protein. Fractions from the column were applied directly onto a 0.45 μm nitrocellulose membrane. Fractions that eluted near the exclusion volume of the column showed the most intense staining in the receptor-binding slot blot. Those fractions were pooled and an aliquot was dialyzed against 40 mM Tris HCl, 1 M NaCl buffer and used in the DDR receptor autophosphorylation assay. Another aliquot was dialyzed extensively against 12 mM HCl and used for a quantitative amino acid analysis.

Purification of Commercially-Derived Collagen V

Two milligrams of human placental collagen type V (Sigma) was dissolved in 25 mM Tris HCl (pH 8.2) containing 50 mM NaCl and 2 M urea and loaded onto a 5 ml Fractogel EMD SO3 strong cation exchange column. The column was eluted at room temperature with 200 ml of a 0–1.0 M NaCl gradient at 2 ml/min. Fractions were pooled based on the receptor slot-blotting activity, and their purity was analyzed by SDS gel electrophoresis on 4%–20% polyacrylamide gels. Activity eluted between 0.4 and 0.5 M NaCl. Active fractions were pooled, dialyzed to reduce NaCl concentration, and injected onto a Pharmacia 1.6 × 5 MonoS PC cation exchange column. The protein was eluted from the column with a sharp gradient of NaCl in a final volume of 0.08 ml. The sample was then run at 4°C on a 3.2 × 30 Superose 6 PC gel filtration column equilibrated in 40 mM Tris HCl pH 8.2 buffer containing 6 M urea. The sample was dialyzed against 40 mM Tris HCl, 1 M NaCl buffer for use in the DDR receptor phosphorylation assay.

Direct Red Assay for Collagen Quantitation

Slot blots or dot blots were prepared as for Rbody-blotting procedure. Blots were either first blotted with Rbody or directly transferred to a 0.1% solution of Direct Red (Sirius Red) dye in saturated picric acid and incubated for 12 hr at room temperature. Blots were washed with 10 mM HCl until washes showed no yellow color. Blots were washed with Mili-Q water and allowed to air dry. Intensity and location of collagen-containing samples were noted qualitatively, or spots were cut out of membrane, placed in a microfuge tube containing 100 μl of 100 mM NaOH, vortexed occasionally during a 30 min period, and the absorbance determined at 570nm of the colored extract. Absorbances of extracts from samples were compared to those from a standard curve of blotted Vitrogen 100 in a range from 0–10μg/well.

Protein Assays

To samples plus enough Mili-Q water to make 0.9 ml was added 0.1 ml. Bio-Rad protein assay dye reagent concentrate. Samples were vortexed on addition of dye reagent and allowed to stand at room temperature for 10–20 min. Absorbances at 595nm were read and compared to standard curves of 0–20 μg/assay bovine serum albumin or Vitrogen 100. Standard curves were found to coincide for protein concentrations up to 10 μg/assay, after which the response of Vitrogen 100 decreases.

Tyrosine Phosphorylation Assays

COS cells were plated 24 hr before transfection in 10 cm tissue culture plates at 10^6 cells/plate, then transfected with 5 micrograms of pCMX-DDR1-myc3 construct using a DEAE transfection protocol as described (Davis et al., 1994), and maintained in 10%BCS/DMEM after transfection. The pCMX-Nep-myc3 construct encodes a full-length DDR1 receptor fused to three consecutive Myc epitope tags at its carboxyterminus. Two days after transfection, the cell media was removed and replaced with DMEM lacking serum. The next day the serum-starved cells were stimulated for 1 hr at 37°C with collagens by adding 0.1 ml of appropriate amounts of collagen in 1% acetic acid; for controls, 1% acetic acid lacking collagen was added. At various times after collagen challenge, the cells were lysed in 1% NP-40 in PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.14 U/ml aprotinin, 1 mM EDTA, and 1 mM sodium orthovanadate. The lysates were immunoprecipitated with lectin from Triticum vulgaris conjugated with agarose macrobeads (Sigma) and immunoblotted with the phosphotyrosine-specific monoclonal antibody 4G10 (1:5000, Upstate Biotechnology). In each case, the blots were stripped with glycine strip buffer and subsequently immunoblotted with the Myc-specific 9E10 antibody to control for DDR1 receptor levels. Immobilized collagen-coated plates for phosphorylation assays were created by soaking plates in 0.5% acetic acid for 20 min at 60°C, rinsing with distilled water, and incubating with 0.1 mg/ml of collagen solution in 0.1M acetic acid overnight at room temperature. The next day the plates were washed with DMEM and dispersed cells (the serum-starved cells prepared as above but removed from plates via trypsin treatment) were added for the phosphorylation assays.

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