Oligomerization-dependent Regulation of Motility and Morphogenesis by the Collagen XVIII NC1/Endostatin Domain✪

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Abstract. Collagen XVIII (c18) is a triple helical endothelial/epithelial basement membrane protein whose noncollagenous (NC)1 region trimerizes a COOH-terminal endostatin (ES) domain conserved in vertebrates, *Caenorhabditis elegans* and *Drosophila*. Here, the c18 NC1 domain functioned as a motility-inducing factor regulating the extracellular matrix (ECM)-dependent morphogenesis of endothelial and other cell types. This motogenic activity required ES domain oligomerization, was dependent on rac, cdc42, and mitogen-activated protein kinase, and exhibited functional distinction from the archetypal motogenic scatter factors hepatocyte growth factor and macrophage stimulatory protein. The mo-

tility-inducing and mitogen-activated protein kinase–stimulating activities of c18 NC1 were blocked by its physiologic cleavage product ES monomer, consistent with a proteolysis-dependent negative feedback mechanism. These data indicate that the collagen XVIII NC1 region encodes a motogen strictly requiring ES domain oligomerization and suggest a previously unsuspected mechanism for ECM regulation of motility and morphogenesis.

Key words: collagen XVIII • endostatin • motility • morphogenesis • extracellular matrix

Introduction

The extracellular matrix (ECM)[1] consists of a protein and carbohydrate substratum comprising the intercellular space not occupied by cell–cell adhesive complexes. Far from a passive support, the ECM has been linked to multiple activities, including structural or adhesive support, growth factor sequestration, and regulation of cell growth, differentiation, and morphogenesis. Proteinaceous ECM components include fibronectin, laminins, vitronectin, and collagens, many of which bind to various αβ integrins with

transduction of intracellular signals (Giancotti and Ruoslahti, 1999; Streuli, 1999).

The collagens comprise a major category of intrinsic ECM proteins, encompassing ~20 family members united by triple helical structure and Gly-Xaa-Yaa repeats. Many collagen functions have been deduced from mutant phenotypes in humans or homozygous knockout mice, including osteogenesis imperfecta, Alport syndrome, Ehlers-Danlos syndrome, and several chondrodysplasias (Prockop and Kivirikko, 1995). On the other hand, collagens are ligands for transmembrane signaling proteins such as the α1β1, α2β1, and α10β1 integrins and discoidin domain receptor (DDR) transmembrane tyrosine kinases which activate downstream responses such as tyrosine phosphorylation and activation of focal adhesion kinase and phospholipase Cγ (Vogel, 1999).

The multiplexin subclass of collagens, represented by collagens XV and XVIII (c15, c18), are characterized by multiple interrupted triple helical regions and a unique COOH-terminal endostatin (ES) domain (discussed below) (Oh et al., 1994; Rehn and Pihlajaniemi, 1994).

Collagen XV/XVIII exhibits striking evolutionary conservation in vertebrates, *Caenorhabditis elegans* and *Drosophila*, with sequencing of the *C. elegans* and *Drosophila* genomes revealing the presence of only type IV and type XV/XVIII verterbrate collagens, as described in this issue by Ackley et al. (2001) and in recent reviews (Hutter et al., 2000; Hynes and Zhao, 2000). The *C. elegans* and *Drosophila* homologues more closely resemble collagen XVIII than collagen XV, based on isoform structure and motifs in a putative heparin-binding domain (Sasaki et al., 2000; Ackley et al., 2001).

Collagen XVIII (c18) is a ubiquitous component of endothelial and epithelial basement membranes (Muragaki et al., 1995; Musso et al., 1998; Saarela et al., 1998) and has been identified recently as the genetic lesion in Knobloch syndrome, an autosomal recessive condition characterized by scalp and/or neural tube closure deficits and retinal degeneration (Sertie et al., 2000). In addition to triple helical repeats, the c18 gene contains an alternative exon encoding a domain homologous to the *Drosophila* tissue polarity gene *frizzled* (see Fig. 1 A) (Rehn et al., 1998; Saarela et al., 1998). The 38-kD COOH-terminal extent of c18 is nontriple helical and is designated noncollagenous (NC)1 domain. Within NC1, a nontriple helical trimerization domain redundantly trimerizes the globular COOH-terminal 20-kD ES domains (see Fig. 1 A) (Sasaki et al., 1998). The NC1 domain represents a predominant tissue form of c18 in numerous tissues, including liver and lung, and is particularly amenable to biochemical study, since it is easily produced as soluble recombinant protein (Sasaki et al., 1998) as opposed to full-length collagen XVIII.

The ES domain within c18 NC1 has been reported to inhibit endothelial proliferation and migration, induce endothelial apoptosis, and inhibit tumor growth (O'Reilly et al., 1997; Dhanabal et al., 1999a,b; Yamaguchi et al., 1999; Dixelius et al., 2000). In vertebrates, the ES domain is only represented in collagen XVIII and the related collagen XV (Muragaki et al., 1994; Sasaki et al., 1998) and conserved (45% amino acid identity) in *C. elegans* and *Drosophila* collagen XVIII homologues. The "hinge" region between the trimerization domain and the ES domain (Fig. 1 A) is cleaved by elastase and/or cathepsin L to release free ES from c18 (Sasaki et al., 1998; Wen et al., 1999; Felbor et al., 2000). It is relevant to the present studies that physiologic proteolysis in the hinge region converts ES from an obligately trimerized form into a monomeric form, as was initially purified (O'Reilly et al., 1997).

In the current work, we describe that the collagen XVIII NC1 domain regulates ECM-dependent motility and morphogenesis of both endothelial and nonendothelial cells in a manner strictly requiring ES domain oligomerization and the activities of rac, cdc42, and the mitogen-activated protein (MAP) kinase (MAPK) pathway. We additionally demonstrate that the motogenic and morphogenic properties of the c18 NC1 trimer are distinct from and in fact antagonized by its physiologic cleavage product ES monomer, and that the c18 NC1 trimer is inhibited by ES monomer in a potential negative autoregulatory loop. Ackley et al. (2001) in this issue describe mechanistically similar findings in which the *C. elegans* collagen XVIII homologue CLE-1 promotes neuronal motility through its trimerization-competent NC1 domain, whereas CLE-1 ES monomers behave as dominant negative (DN) alleles which inhibit motility induced by wild-type CLE-1. These data implicate domains of the evolutionarily conserved type XVIII collagen in the regulation of cell motility and establish a previously unsuspected mechanism for ECM regulation of motility and morphogenesis.

Materials and Methods

Expression of Recombinant Proteins

Human Fc-human ES proteins, containing the 184–amino acid COOH-terminal ES domain from HSHRDF through SFMTASK, were purified from conditioned medium of stable myeloma transfectants and human ES monomer produced by subsequent enterokinase (EK) cleavage as described previously (Ding et al., 1998; Lo et al., 1998). Disulfide-bonded human ES dimer was produced similarly except that the expression vector pdCs-Fc(D4K)-huEndo was mutated Q7→C7 to produce pdCs-Fc(D4K)-huEndo(C7).

The human collagen XVIII NC1 was produced by PCR with primers 5′-GATC GGCC CAGCC GGCC CAT CAT CAC CAT CAC CAT GAC GAT GAC GAT AAG TCA GGG CAG GTG AGG CTC TGG GCT ACA CGC CAG G-3′ and 5′-GATC GGATCC CTA CTT GGA GGC AGT CAT GAA GC-3′ using human collagen XVIII cDNA (a gift from B. Olsen, Harvard Medical School, Boston, MA) as template amplifying SGQVRL through the COOH terminus. Murine collagen XVIII NC1 was produced identically using the primers 5′-GATC GGCC CAGCC GGCC CAT CAT CAC CAT CAC CAT GAC GAT GAC GAT AAG GCT GGG CAG GTG AGG ATC TGG GCC ACA TAC C-3′ and 5′-GATC GGATCC CTA TTT GGA GAA AGA GGT CAT GAA GC-3′ from the template mc3b (a gift from B. Olsen), amplifying AGQVRI through the COOH terminus. Murine collagen XV NC1 was amplified from mc15 cDNA (a gift from T. Pihlajaniemi, University of Oulu, Oulu, Finland) with 5′-GATC GGCC CAGCC GGCC CAT CAT CAC CAT CAC CAT GAC GAT GAC GAT AAG GTC ACA GGC CTC AGC GAC ATG GGT GAC ATG CTA CAG-3′ and reverse primer 5′-GATC GGATCC TCA CTT CCT AGT GTC TGT CAT GAA AC-3′, amplifying LSDM through the COOH terminus. The PCR products hNC1(c18), mNC1(c18), or mNC1(c15) were cloned SfiI-BamHI into pSecTag2A (Invitrogen) in frame with the IgK signal peptide, transfected into 293T cells by calcium phosphate, and conditioned medium-purified over Ni-agarose using the NH₂-terminal 6× His tag embedded by PCR.

Two versions of the ES domain of murine collagen 15 were amplified from mc15 cDNA (a gift from T. Pihlajaniemi) by PCR using the forward primers 5′-GATC C CCG GGT TCA GGG GAT GAC GAT GAC GAT AAG TTA AGT GCC AAC TAT GAG AGG CCT G-3′ or 5′-GATC C CCG GGT TCA GGG GAT GAC GAT GAC GAT AAG CCT GTT CTG CAC CTG GTT GCT CTG-3′ and the reverse primer 5′-GATC CTC GAG TCA CTT CCT AGT GTC TGT CAT GAA AC-3′, amplifying LSANYER or PVLHLVA through the COOH terminus. The resultant fragments were ligated XmaI-XhoI into the expression vector pdCs (Lexigen) in frame with a 5′ coding region for murine IgG2α Fc, and Fc-ES(c15) was purified from conditioned medium of stable myeloma transfectants using protein A–Sepharose. No differences were observed using either of the two Fc-ES(c15) recombinant proteins.

SDS-PAGE Analysis of Purified Recombinant Human c18 Fragments

Human ES monomer from Fc-ES EK cleavage or human ES dimer from EK cleavage of Fc-ES(Q7→C7) (analyzed by SDS-PAGE at 5 μg/lane under nonreducing [without DTT] or reducing [with DTT] conditions). The doublet observed with hES monomer represents a four–amino acid NH₂-terminal cleavage. Human NC1 ES trimer (40 μg) with or without ethylene glycol bis-succinimidyl succinate (EGS) cross-linking (see Materials and Methods) was analyzed by SDS-PAGE.

Cell Culture and Lines

Human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells were purchased from Clonetics and grown in EBM containing EGM-2-MV supplements (vascular endothelial growth factor [VEGF], hepatocyte growth factor [HGF], and hydrocortisone). 293 cells and MDCK cells were obtained from American Type Culture Collection and grown in DME with 10% FCS and L-glutamine. PC12 cells were a gift from M. Greenberg (Children's Hospital, Boston, MA)

and grown in DME with 10% horse serum, 5% FCS, penicillin streptomycin, and L-glutamine on collagen I–coated dishes.

Matrigel Tube Formation Assay

Matrigel, an ECM preparation from the Engelbreth-Holm-Swarm tumor, was purchased from Collaborative/BD PharMingen. Multiwell dishes (24-well) were coated with 250 μl Matrigel at 4°C and incubated at 37°C for 20 min followed by seeding with HUVECs, human dermal microvascular endothelial cells, or other cell types in 1 ml complete medium at 50,000 cells/ml. Recombinant ES monomer, dimer, NC1, or Fc-ES fusion proteins were added either at plating or after 16 h of tube formation. Molecular masses used for calculation of molarity were as follows: ES monomer (20 kD), ES dimer (40 kD = 20 × 2), NC1(c18) trimer (114 kD = 38 × 3), and NC1(c15) trimer (93 kD = 31 × 3). Cells were photographed under phase–contrast and images were imported into Adobe Photoshop® or Canvas 5. Where relevant, cells were preincubated with ES monomer (30 min) or *Clostridium difficile* toxin B (50 ng/ml, 4 h) before addition of hNC1 or ES dimer. HGF (R&D Systems) was used at 50 ng/ml. Tubes were quantitated by manual counting in triplicate of low power fields (40×). Phalloidin-FITC staining for actin was performed after paraformaldehyde fixation using confocal microscopy.

Assay for MAPK Activation

HUVECs on Matrigel for 16 h were preincubated for 60 min with or without PD98056 or SB203580 (50 μM) (Calbiochem), followed by treatment with hES monomer (3,000 nM), hES dimer (50 nM), or c18 NC1 (50 nM). Cells were washed twice in Hepes-buffered saline and lysed with SDS-PAGE sample buffer with β-mercaptoethanol. Lysates were analyzed by SDS-PAGE and Western blotting using anti–phospho-MAPK or extracellular signal–regulated kinase (ERK)2 antisera (New England Biolabs, Inc.). Fold activation was calculated by densitometry of anti–phospho-MAPK Western blots, assigning activity of unstimulated extracts as one-fold. No significant differences were observed between untreated and monomer-treated cells.

Adenoviral Infection of Endothelial Tubules

Recombinant adenoviruses encoding a chimeric tetracycline repressor/VP16 transactivator domain (Ad–tTA) or encoding DN alleles of rac or rho under the control of a minimal cytomegalovirus promoter driven by seven tetracycline operator sites have been described previously (Kalman et al., 1999). The construction of Ad-cdc42DN will be described elsewhere. HUVECs were infected at the time of plating on Matrigel in complete medium with Ad-tTA at a multiplicity of infection of 1:1,000. After 12 h, tubule formation was identical to uninfected cells (data not shown), and tubules were infected as appropriate with Ad-racDN, Ad-rhoDN, or Ad-cdc42DN for 4 h at a multiplicity of infection of 1:2,000. Subsequently, cells were treated with hES dimer (75 nM) for 24 h and photographed under phase–contrast. All incubations were in the absence of tetracycline to allow for maximal transactivation of the Ad-DN constructs. Inhibition of motility was not observed with either Ad-tTA or Ad-DN viruses alone (data not shown).

Online Supplemental Material

HUVECs were plated into Matrigel-coated 2-well slide chambers as above, and tubes were allowed to form for 16 h. Human ES dimer (50 nM) was added at the start of videomicroscopy (see Fig. 3 C; video available at http://www.jcb.org/cgi/content/full/152/6/1233/DC1). The total video length is 12 h and was captured in a total of 360 frames at 30 frames per hour. Images were captured on a phase–contrast microscope at 200× magnification, saved to disk, and converted to avi format using Image Pro Plus software. The video was converted to Quicktime® 4 format using Vid4Win 2 QT software (shareware).
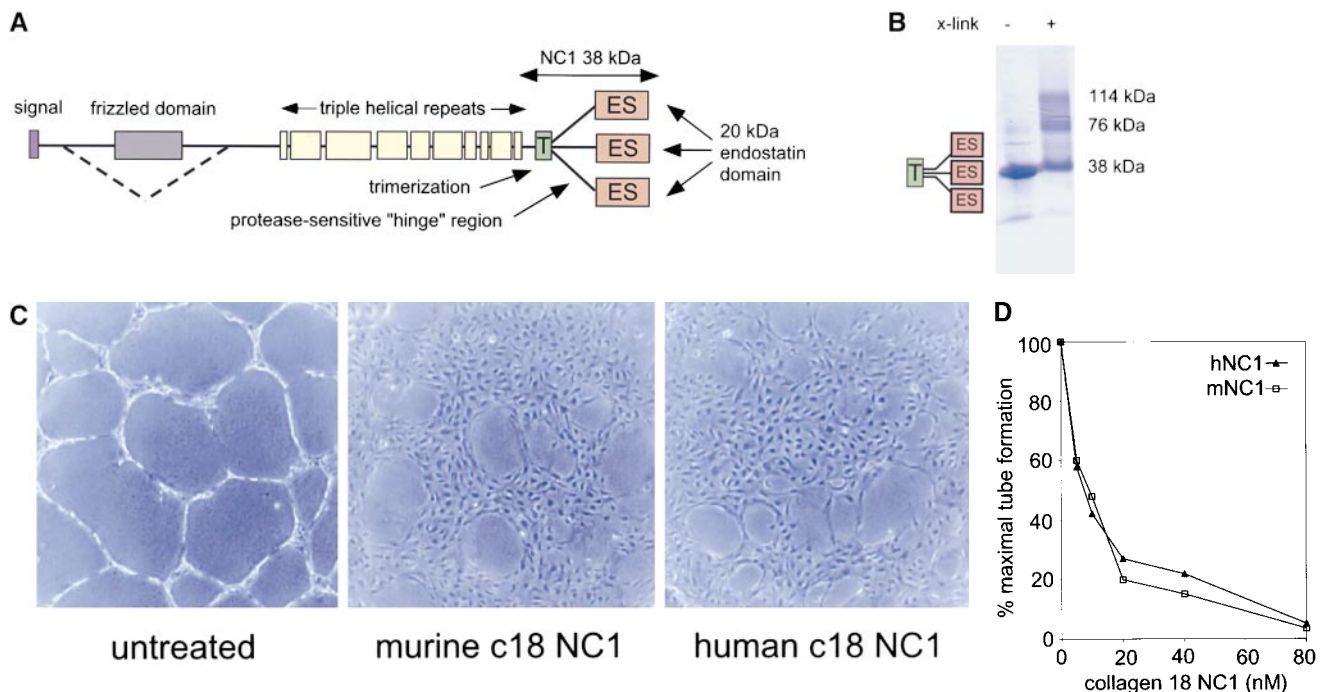

Figure 1. Inhibition of in vitro endothelial tube assembly by collagen XVIII NC1. (A) Topology of collagen XVIII. Signal peptide, triple helical collagenous repeats, NC1 domain, and a protease-sensitive "hinge" region interposed within the trimerization and ES motifs are indicated. Dotted line indicates alternative splice which occurs in nonhepatic tissues and removes the frizzled homology domain. (B) Production of recombinant c18 NC1. Human c18 NC1 purified from supernatant of stably transfected 293T cells was analyzed by SDS-PAGE with or without EGS cross-linking. Oligomeric forms corresponding to NC1 trimer at ∼114 kD (38 × 3) and 76-kD species from incomplete cross-linking (38 × 2) are indicated. (C) Recombinant c18 NC1 inhibits in vitro endothelial tube formation. HUVECs were plated on Matrigel in the absence (untreated) or presence of murine or human c18 NC1 at 50 nM and photographed under phase–contrast after 16 h (magnification ×40). A complete lack of tubular structures and a dispersed cell phenotype are apparent in the NC1-treated wells. (D) Dose-dependent inhibition of endothelial tube formation by recombinant c18 NC1. Indicated amounts of human or mouse c18 NC1 were added at the time of HUVEC seeding onto Matrigel-covered wells. Tubular structures were quantitated by manual counting of low power fields after 16 h and percent inhibition was expressed using untreated wells as 100%.
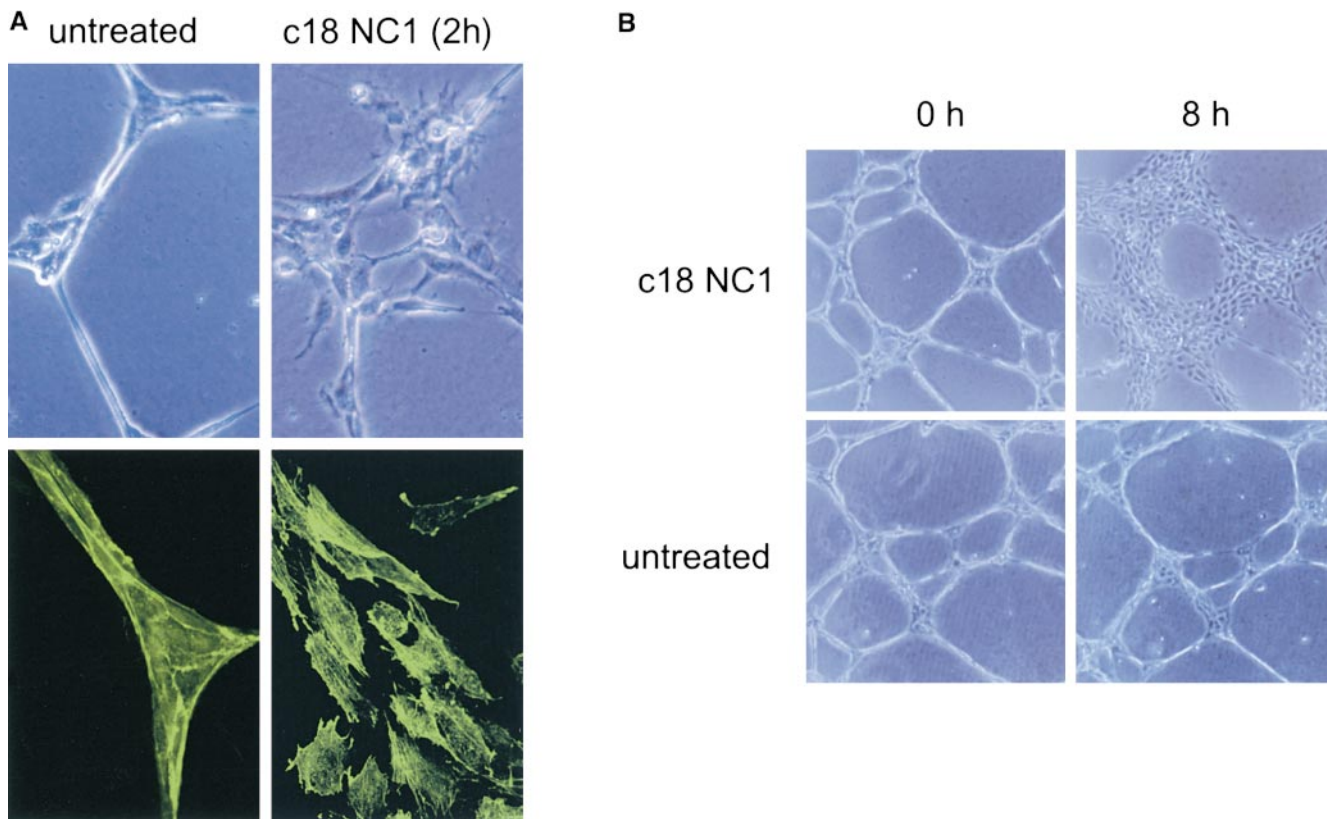
Figure 2. Stimulation of endothelial cell motility by collagen XVIII NC1. (A) Rapid induction of motility by human c18 NC1. HUVECs were plated on Matrigel for 16 h and allowed to form tubular structures, followed by recombinant human c18 NC1 treatment (50 nM) for 2 h. Top, phase–contrast microscopy (magnification ×200); bottom, confocal microscopy. Cells treated as above were stained with phalloidin-FITC to stain filamentous actin and photographed under confocal microscopy. NC1-treated cells exhibit prominent cell migration with formation of stress fibers, filopodia, and lamellipodia. (B) Migration of HUVECs from preestablished tubules is stimulated by c18 NC1. HUVECs were plated on Matrigel for 16 h and identical fields were photographed under phase–contrast before (0 h) and after (8 h) stimulation by recombinant human c18 NC1 (50 nM) (magnification ×40).

Results

Inhibition of Endothelial Tube Formation by the Collagen XVIII NC1 Domain

To evaluate potential functions of collagen XVIII, the soluble c18 NC1 domain was produced from conditioned medium of transfected 293T cells and migrated at 38 kD upon SDS-PAGE (Fig 1 B, left lane) in agreement with published results (Sasaki et al., 1998). Consistent with trimerization, this recombinant protein produced an ~114-kD species (38 × 3) upon EGS cross-linking (Fig. 1 B, right lane), confirming previous reports documenting an NH_2-terminal trimerization domain, migration as a trimer upon gel filtration, and trimeric appearance upon electron microscopy (Sasaki et al., 1998).

We explored the behavior of c18 NC1 in an in vitro morphogenesis assay during which endothelial cells plated on ECM preparations, such as Matrigel, spontaneously aggregate and assemble into densely multicellular capillary-like tubular structures (Folkman and Haudenschild, 1980; Grant et al., 1991). Although when used with endothelial cells, this assay recapitulates numerous aspects of angiogenesis, including cellular migration, differentiation, and metalloproteinase activation (Grant et al., 1991), nonendothelial cells such as fibroblasts can undergo similar morphologic changes, and thus this assay may best reflect ECM-induced changes in cell adhesion and morphogene-

sis. Human or murine c18 NC1 treatment at the time of HUVEC plating on Matrigel strongly inhibited assembly into tubular structures, with cells remaining dispersed and exhibiting a morphology resembling cells on plastic rather than aggregating into characteristic capillary-like tubes (Fig. 1 C). Quantitation of tubular structures by light microscopy revealed a dose-dependent effect with an IC_{50} of ~10 nM (Fig. 1 D). Stimulation of proliferation was not detected by BrdU staining or [^{3}H]thymidine incorporation during this time interval, nor was stimulation of cell death detected after recovery of cells from Matrigel and trypan blue staining (data not shown). Identical results were obtained with primary cultures of human microvascular, umbilical artery, or pulmonary artery endothelial cells, and morphological effects were not observed on tissue culture plastic or on gels of purified matrix proteins such as collagen I or fibrin (data not shown).

Induction of Cellular Motility by the Collagen XVIII NC1 Domain

The dispersed phenotype of HUVECs treated with c18 NC1 prompted investigation of the effects of this protein on preestablished endothelial tubules. Strikingly, when applied to HUVEC tubules, recombinant c18 NC1 stimulated active cell motility with migration of cells away from the structures easily observable by 2 h of treatment (Fig. 2 A, top). Phase–contrast microscopy revealed abun-
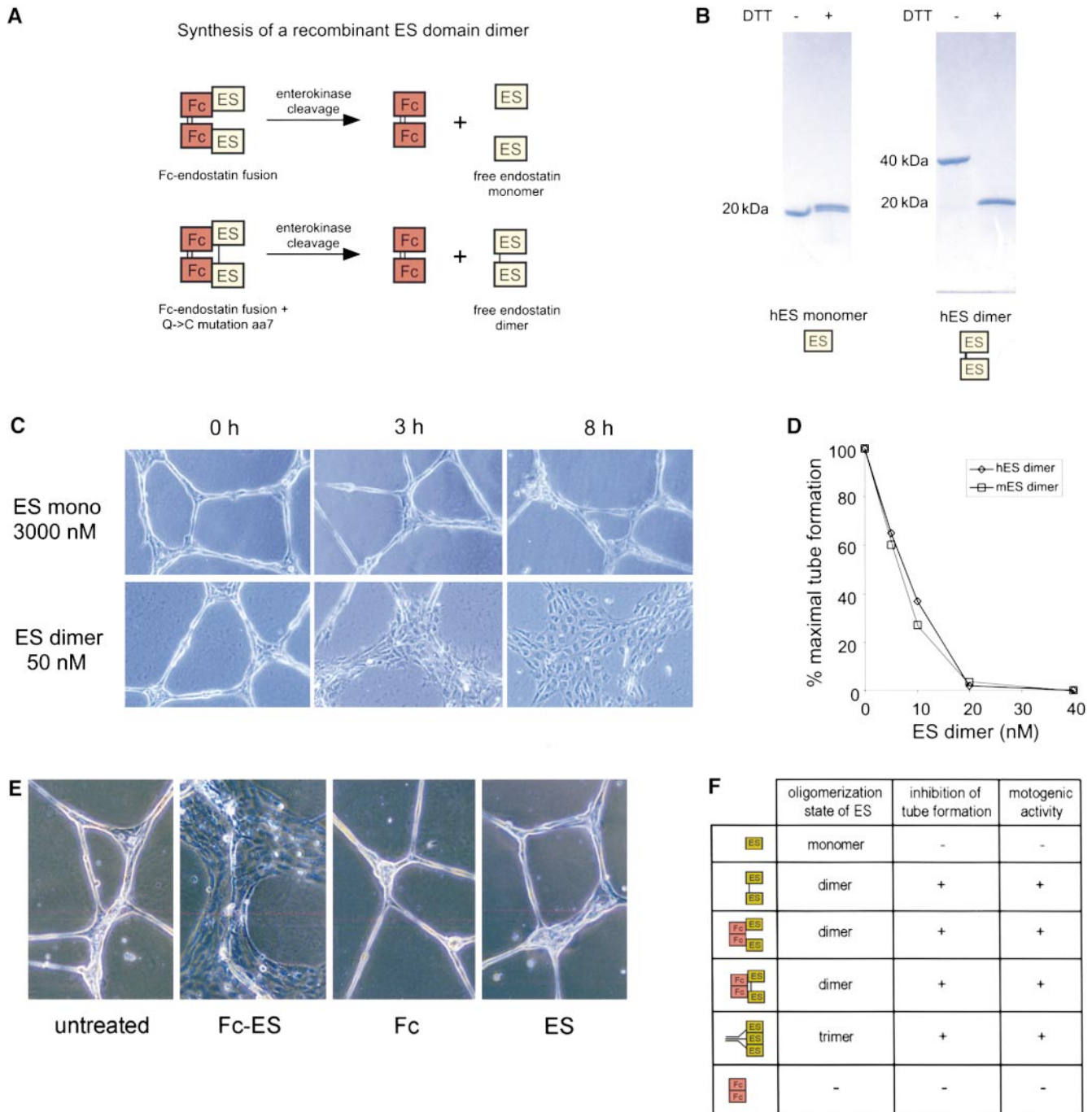
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Figure 3. ES domain oligomerization is necessary and sufficient for c18-induced motility. (A) Schematic of synthesis of ES domain monomer and dimer. Cleavage of an Fc-ES domain fusion produces free ES monomer. In contrast, cleavage of Fc-ES(Q7→C7) produces an ES domain dimer because of disulfide bond formation between adjacent C7 residues. (B) Nonreducing and reducing SDS-PAGE of purified recombinant human ES monomer and dimer. Migration shift from 40 to 20 kD is noted for ES dimer but not ES monomer upon disulfide reduction with DTT. (C) ES dimer but not ES monomer stimulates migration of HUVECs from pre-formed tubules on Matrigel. HUVECs were plated on Matrigel and allowed to form tubes for 16 h, followed by stimulation with ES monomer (3,000 nM) or ES dimer (50 nM) for the indicated times. Representative fields were photographed under phase–contrast (magnification ×200). (D) Dose response to recombinant human ES dimer. HUVECs were plated on Matrigel in the presence of hES dimer, and after 16 h tubular structures were quantitated by manual counting of central fields and percentage of maximal tube formation was calculated. (E) Induction of motility by Fc-ES fusions which dimerize the ES domain. Preformed HUVEC tubules were treated with Fc-mES(c18) (40 nM), Fc (3,000 nM), or mES(c18) (3,000 nM) and photographed after 30 h (magnification ×40). (F) Summary of motogenic activity of monomeric, dimeric, and trimeric ES domain derivatives.

dant structures resembling filopodia and lamellipodia, which were confirmed upon confocal microscopy for phalloidin-FITC reactivity (Fig. 2 A, bottom) and by videomicroscopy (see below). This cellular migration was more pronounced with time, with cells becoming progressively more dispersed and acquiring a "scattered" phenotype (Fig. 2 B). Preformed tubules were increasingly refractory to dispersion after 16–24 h and moto-
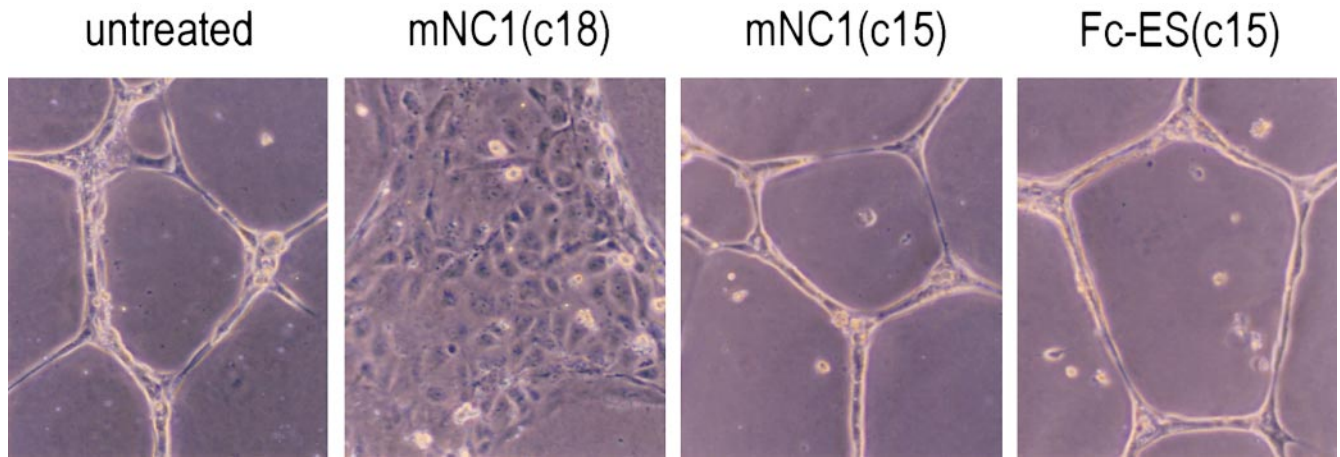
Figure 4. Oligomerized collagen XV ES domains do not exhibit motogenic activity. HUVECs were plated on Matrigel in the presence of mNC1(c18) (50 nM), mNC1(c15) (100 nM), or Fc-mES(c15) (250 nM) and photographed after 20 h. Identical results were obtained upon application of factors to preformed tubules.

genic activity was not observed against HUVECs plated on plastic (data not shown).

ES Domain Oligomerization Is Necessary and Sufficient for Motogenic Activity

To localize the motogenic region of c18 NC1, a series of derivative proteins were constructed. The potential involvement of the ES domain comprising the COOH-terminal 184 amino acids of NC1 (Sasaki et al., 1998) was examined with an Fc-ES domain fusion protein (Fc-ES) containing an NH$_2$-terminal human IgG1 Fc fragment, an interposed EK cleavage site, and a COOH-terminal ES domain (Fig. 3 A). EK cleavage of Fc-ES allowed large scale production of ES domains (Fig. 3 A) whose composition and monomeric nature were confirmed by denaturing and nondenaturing SDS-PAGE (Fig. 3 B, left), and by gel

filtration and mass spectrometry (data not shown). Human and murine ES monomers thus produced were completely inactive at eliciting motility at 3,000 nM, the highest concentration tested (Fig. 3 C, top), as were murine and human ES monomers produced in baculovirus or yeast (data not shown).

Since the ES domain is physiologically trimerized in the context of collagen XVIII and NC1 (Sasaki et al., 1998) (Fig. 1 A), we evaluated whether c18 motogenic activity required ES domain oligomerization by constructing an artificial ES domain dimer. The ES crystal structure predicts a dimer in which glutamine-7 residues in adjacent subunits are in close proximity (Ding et al., 1998). Fusion of heterologous proteins to an antibody Fc fragment produces obligate dimerization via disulfide bonds in the Fc region (Lo et al., 1998). Mutation of ES glutamine-7 residue to cys-
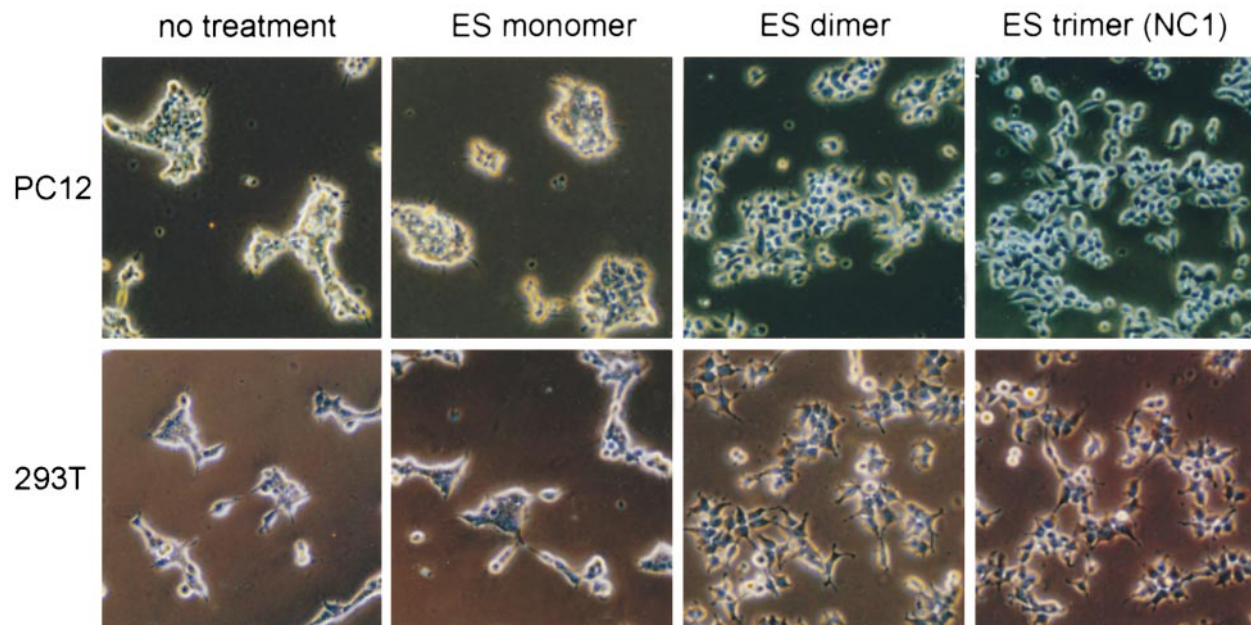

Figure 5. Motile response of nonendothelial cell types to c18 derivatives. PC12 and 293T cells were cultured with hES monomer (3,000 nM), hES dimer (25 nM), or hNC1 (50 nM) on Matrigel for 16 h and photographed under phase–contrast (magnification ×200 [bottom]).
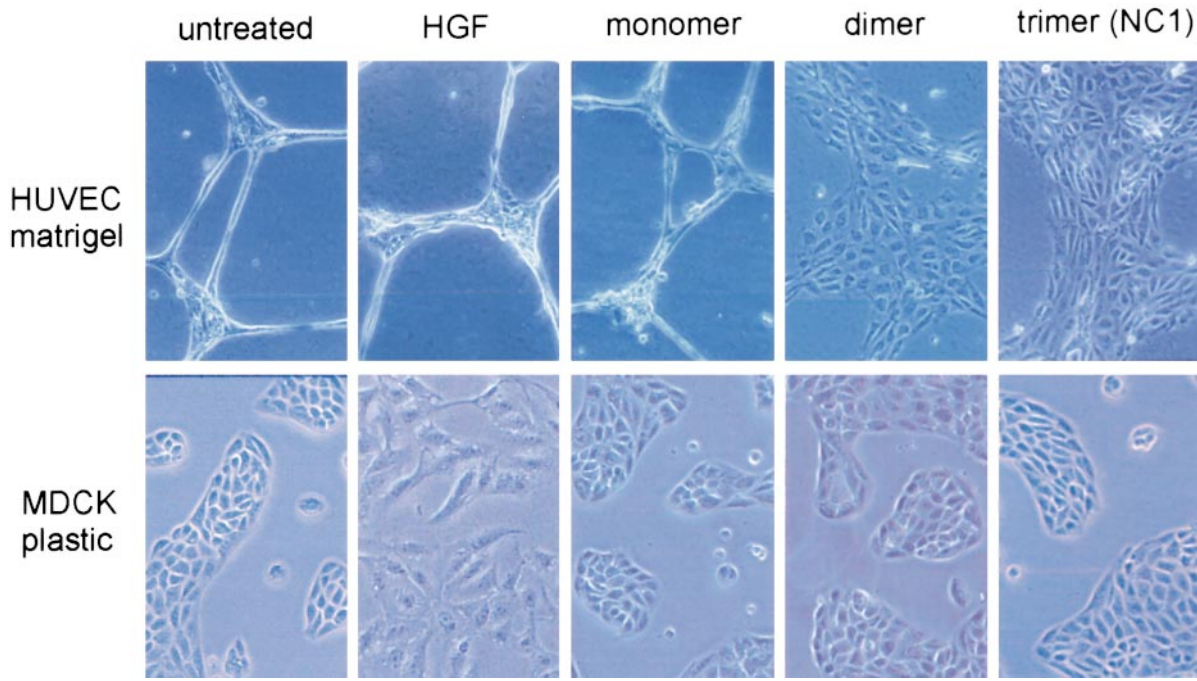
Figure 6. Comparison of c18 and HGF motogenic activities. HUVEC tubules on Matrigel for 18 h (top) or MDCK cells on plastic (bottom) were treated with HGF (50 ng/ml), hES monomer (3,500 nM), hES dimer (50 nM), or c18 NC1/hES trimer (50 nM) for 24 h and photographed. Top, magnification ×200; bottom, ×100. Note complete absence of HGF response in HUVECs on Matrigel and complete absence of c18 responses in MDCK cells on plastic.

teine in the context of Fc-ES fortuitously produced a novel intermolecular disulfide bond between adjacent ES molecules (Fig. 3 A, bottom). EK cleavage of this mutant Fc-ES(Q→C) then liberated free ES dimer migrating at 40 and 20 kD under nonreducing and reducing conditions, respectively (Fig. 3, A and B, right). This ES domain dimer potently reproduced the inhibitory activity of the Fc-ES fusion or NC1 with half-maximal inhibition at ~10 nM (Fig. 3, C and D). Videomicroscopy of endothelial tubules treated with ES dimer confirmed rapid (<2 h) stimulation of motility, formation of filopodia and lamellipodia, and virtual absence of cell division (see video at http://www.jcb.org/cgi/content/full/152/6/1233/DC1), paralleling findings with c18 NC1 (Figs. 1 and 2). The activity of the ES dimer (10 nM) versus the inactivity of the ES monomer (>3,000 nM, the highest concentration tested) indicated that the active region of c18 NC1 localized to the ES domain and that oligomerization of the ES domain was both necessary and sufficient for motogenic activity. Further consistent with a requirement for oligomerization, the Fc-ES (wt) protein, in which the ES domain is obligately dimerized, potently stimulated motility from preformed HUVEC tubules, whereas either Fc or ES derived from EK cleavage of Fc-ES was ineffective (Fig. 3 E). Identical stimulation of motility was observed with Fc-ES(Q→C) (data not shown). The activity of c18 derivatives in which the ES domain is oligomerized, versus the inactivity of ES monomers is summarized (Fig. 3 F).

Oligomerized Collagen XV ES Domains Do Not Exhibit Motogenic Activity

Collagen XVIII and collagen XV define the multiplexin subclass of collagens, characterized by multiple interrup-

tions in the triple helical region and a globular COOH-terminal ES domain. The NC1 domains of both c18 and c15 trimerize (Sasaki et al., 2000), and the cognate ES domains exhibit 60% amino acid identity (Muragaki et al., 1994; Oh et al., 1994; Rehn and Pihlajaniemi, 1994). Despite these similarities, the c18 and c15 NC1 domains differ strikingly in their abilities to associate with heparin and zinc, in affinity for matrix components exhibited by monomers versus trimers, and a shorter "hinge" region in the NC1 domain in c15 (Sasaki et al., 2000). We produced recombinant c15 NC1 domains from the conditioned medium of transfected 293T cells but observed complete lack of inhibition of HUVEC tube formation on Matrigel at 100 nM, the highest concentration tested (Fig. 4). Similarly, dimeric Fc fusion proteins containing Fc at the NH_2 terminus and the collagen XV ES domain at the COOH terminus (Fc-ES[c15]) neither scattered endothelial tubules (Fig. 4) nor antagonized the effects of Fc-ES(c18) at 30-fold molar excess (data not shown). Under these conditions, the motogenic properties of ES domain oligomers are thus highly specific for c18 and not c15, in accordance with divergent structural and functional properties (Muragaki et al., 1994; Oh et al., 1994; Rehn and Pihlajaniemi, 1994; Sasaki et al., 2000).

Collagen XVIII NC1 Motogenic Activity Is Not Specific for Endothelial Cells

The growth-inhibitory, migration-inhibitory, or apoptosis-stimulatory activities of the monomeric ES domain have been reported to be selective for endothelial cells and not other cell types (O'Reilly et al., 1997; Dhanabal et al., 1999a,b). On the other hand, the precursor molecule c18 is not only expressed in vascular basement membranes, but also in most epithelial membranes such as liver, lung, and
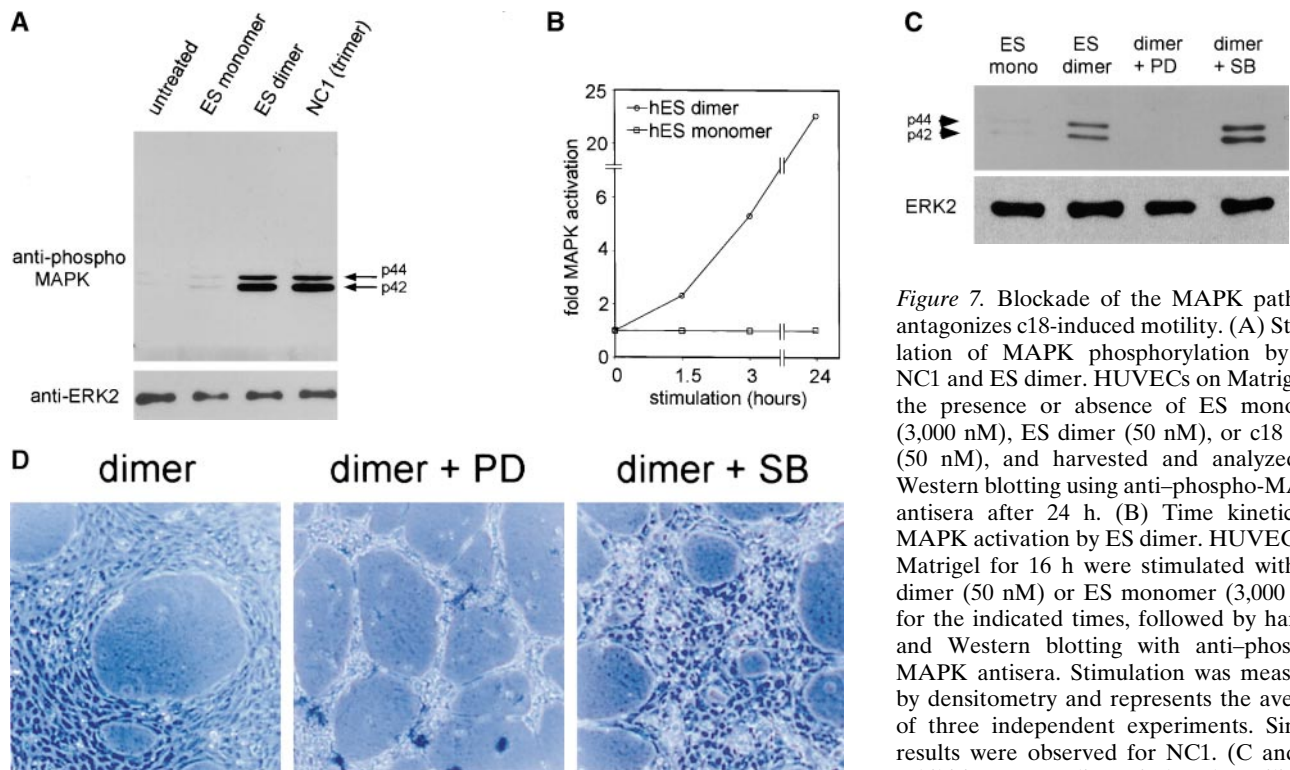
Figure 7. Blockade of the MAPK pathway antagonizes c18-induced motility. (A) Stimulation of MAPK phosphorylation by c18 NC1 and ES dimer. HUVECs on Matrigel in the presence or absence of ES monomer (3,000 nM), ES dimer (50 nM), or c18 NC1 (50 nM), and harvested and analyzed by Western blotting using anti–phospho-MAPK antisera after 24 h. (B) Time kinetics of MAPK activation by ES dimer. HUVECs on Matrigel for 16 h were stimulated with ES dimer (50 nM) or ES monomer (3,000 nM) for the indicated times, followed by harvest and Western blotting with anti–phospho-MAPK antisera. Stimulation was measured by densitometry and represents the average of three independent experiments. Similar results were observed for NC1. (C and D) Inhibition of ES dimer–induced motility by the MEK inhibitor PD98056 but not the p38 inhibitor SB203580. HUVECs were seeded on Matrigel for 16 h followed by 60-min preincubation with or without PD98056 or SB203580 (50 μM), followed by treatment with hES dimer (50 nM) for 12 h, and then harvest for anti–phospho-MAPK Western blot (C) or phase–contrast microscopy (D) (magnification ×40).

kidney, paralleling collagen IV (Muragaki et al., 1995; Musso et al., 1998; Saarela et al., 1998). Furthermore, the clinical spectrum of Knobloch syndrome has implicated collagen XVIII in diverse functions including neural tube and/or scalp closure and maintenance of retinal structure (Sertie et al., 2000), whereas cle-1 mutations in *C. elegans* produce neuronal migration deficits (Ackley et al., 2001).

The ability of c18 NC1 or ES dimer to induce motility of nonendothelial cell types was evaluated using PC12 pheochromocytoma cells or 293T embryonic kidney cells plated on Matrigel. Under these plating conditions, these cell lines aggregated into multicellular collections (Fig. 5). However, culture with ES dimer or c18 NC1 produced a dispersed phenotype resembling plastic-plated cells rather than forming multicellular aggregates, with ES monomer completely inactive (Fig. 5). We have also observed similar results with NIH 3T3 fibroblasts and Lewis lung carcinoma (data not shown). The trimeric c18 NC1 domain can thus modulate the matrix-regulated motility and morphogenesis of numerous cell types and possesses considerably broader tissue tropism than described for monomeric c18 ES domains (O'Reilly et al., 1997; Dhanabal et al., 1999a,b; Yamaguchi et al., 1999).

Functional Distinction between the c18 and HGF/MSP Motogens

The motile response to c18 NC1 resembled the activity of the motogens HGF/scatter factor and macrophage stimulatory protein (MSP) (Gherardi and Stoker, 1991) which regulate motility, morphogenesis, proliferation, and apoptosis. HGF and MSP have been termed "scatter factors" because of their ability to induce motility and disaggregation of cells from acini formed on tissue culture plastic (Gherardi and Stoker, 1991). The motogenic activity of HGF correlates with in vivo augmentation of the metastatic potential of tumorigenic lines (Jeffers et al., 1996a,b) and with essential roles in the developmental migration of muscle and neural crest precursors (Tamagnone and Comoglio, 1997). Additionally, HGF possesses morphogenic activities which promote angiogenesis and tubulogenesis both in vivo and in vitro (Grant et al., 1993; Tamagnone and Comoglio, 1997).

Given the ability of HGF and c18 NC1 to induce motility and disaggregation of cells, effects on HUVEC tube formation were compared. In contrast to c18 NC1 and ES dimers, HGF did not promote motility of HUVECs (Fig. 6, top), despite abundant expression of the HGF receptor c-met on HUVECs (Bussolino et al., 1992). On the other hand, HGF potently disaggregated and scattered MDCK (Fig 6, bottom) and HepG2 cells (data not shown) on plastic, whereas NC1 and ES dimers were inactive under these conditions (Fig. 6, bottom). MSP was also unable to disaggregate HUVEC tubules on Matrigel (data not shown). Notably, HGF stimulates tube formation in collagen matrices (Bussolino et al., 1992; Grant et al., 1993; Tamagnone and Comoglio, 1997), as opposed to the antitubulogenic effects of c18 NC1 described here for endothelial cells. The collagen XVIII NC1 domain therefore defines a novel class of motogenic factor which strictly requires the presence of ECM and is both structurally and functionally distinct from HGF/MSP.
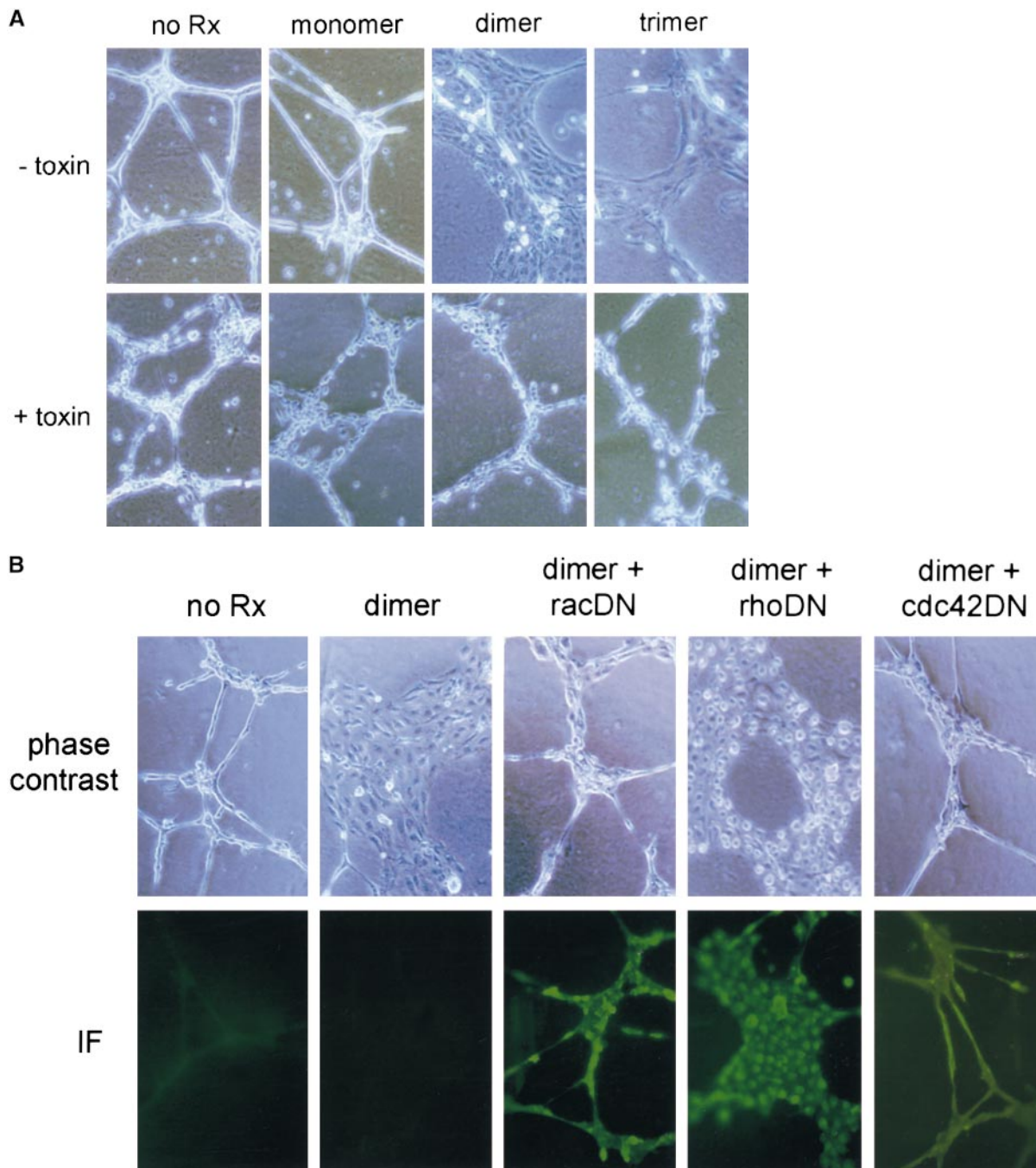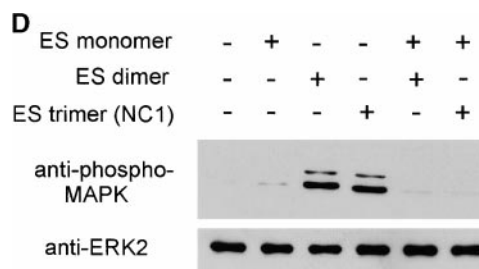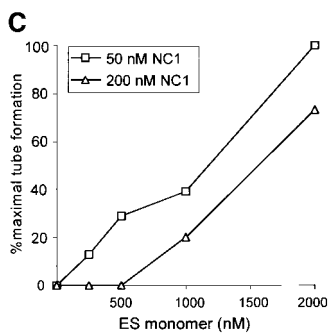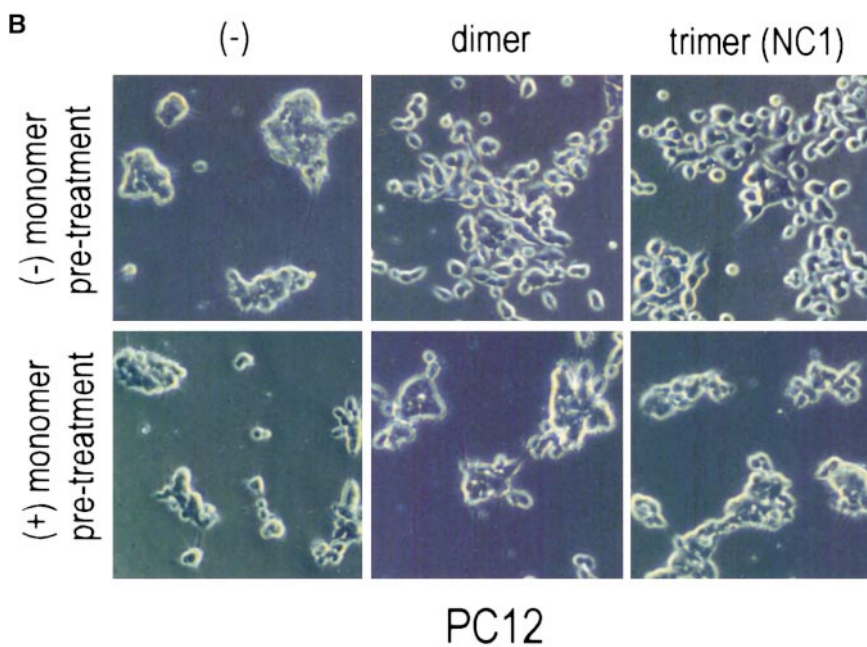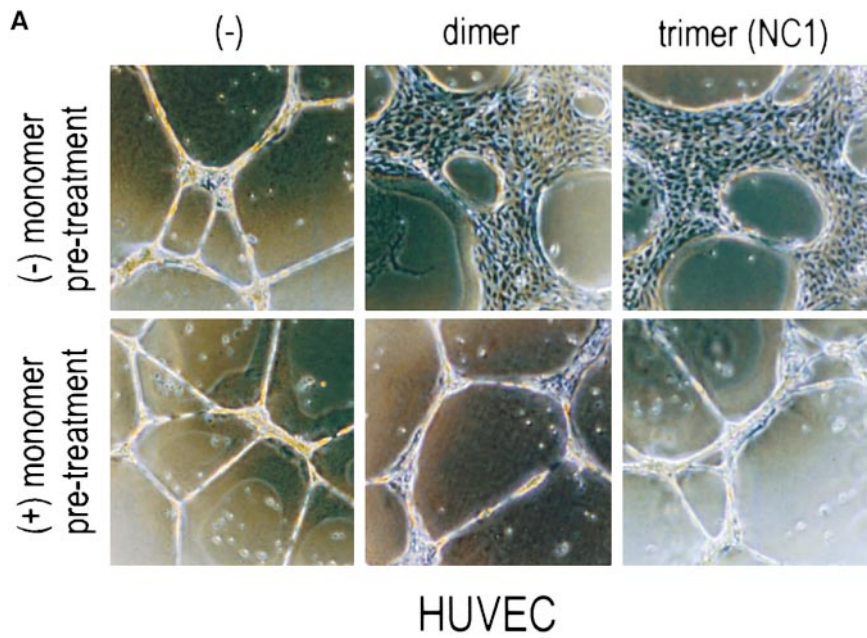
Figure 8. Motogenic activity of c18 NC1 requires rac and cdc42. (A) Inhibition of c18-stimulated motility by *C. difficile* toxin B. HUVECs on Matrigel for 12 h were preincubated with or without *C. difficile* toxin B (50 ng/ml) for 4 h and treated with hES monomer (3,000 nM), hES dimer (50 nM), or c18 NC1 (50 nM) for 16 h, then photographed under phase–contrast (magnification ×200). Trypan blue staining of these cultures revealed <5% toxicity from the toxin treatment. Cell-rounding characteristic of rho inhibition is present in +toxin panels. (B) Inhibition of c18 NC1-stimulated motility by adenovirus-encoded DN alleles of rac and cdc42. Top, HUVEC tubules previously infected with adenovirus encoding racDN, rhoDN, or cdc42DN were treated with hES dimer (75 nM) and photographed (magnification ×200). Strong inhibition of motility by racDN and cdc42DN is present. RhoDN was ineffective at blocking scatter, although cell-rounding characteristic of rho inhibition was observed. Bottom, Parallel cultures were treated identically and processed for immunofluorescence using anti-myc epitope tag (rac, cdc42) or anti-rho (rhoDN and dimer alone), revealing >95% infection with racDN, rhoDN, and cdc42DN.

Collagen XVIII NC1 Requires MAPK Activation for Induction of Motility

The MAPK pathway regulates numerous aspects of cell growth, differentiation, and responses to extracellular stimuli (Schaeffer and Weber, 1999) and is required for HGF-stimulated motility (Potempa and Ridley, 1998). On ECM substrata, only c18 NC1 and ES dimer, and not ES monomer, activated MAPK. MAPK induction was

A

HUVEC

B

PC12

C

D

Figure 9. Autoregulatory inhibition of c18 NC1 by the cleavage product ES monomer. (A) ES monomer inhibits motility induced by c18 NC1 or hES dimer. Left panels, HUVEC tubules on Matrigel for 12 h were treated with ES dimer (50 nM) or NC1/ES trimer (75 nM) for 24 h after 30-min preincubation with human ES monomer (3,000 nM) as appropriate, followed by phase–contrast microscopy (magnification ×40). B, PC12 cells were plated on Matrigel in the presence of ES dimer (50 nM) or NC1/ES trimer (75 nM) for 20 h with or without 30-min preincubation with human ES monomer (3,000 nM) as appropriate, followed by phase–contrast microscopy (magnification ×200). Similar results were obtained with treatment with factors after 12–16-h culture on Matrigel. (C) Dose-dependent reversal of c18 NC1 inhibition of HUVEC tube formation by ES monomer. Increasing concentrations of hES monomer were added to freshly seeded HUVECs on Matrigel, followed after 30 min by addition of c18 NC1 (50 or 200 nM) and quantitation of tube formation after 16 h. Higher concentrations of ES monomer were required to reverse inhibition by 200 nM as opposed to 50 nM NC1. (D) Stimulation of MAPK by c18 NC1 or ES dimer is inhibited by ES monomer. HUVEC tubules on Matrigel for 16 h were preincubated with 3,000 nM hES monomer or PBS for 30 min as appropriate, followed by stimulation with hNC1 or hES dimer (50 nM) for 24 h and Western blotting with anti–phospho-MAPK antibody.

first detectable within 90 min, paralleling the onset of cellular motility, and persisted for >24 h, in contrast to monomer or untreated cultures in which basal MAPK activity declined to undetectable levels at that time (Fig. 7, A and B). These persistent activation kinetics are reminis-

cent of signaling via the DDR collagen receptor kinases, which require 18 h of exposure for maximum induction of phosphotyrosine (Vogel, 1999), and HGF, which also stimulates prolonged (>16 h) MAPK activation (Potempa and Ridley, 1998). Stimulation of tyrosine phos-

phorylation in HUVECs on Matrigel by c18 derivatives also requires ES domain oligomerization (LaMontagne, K.R., and C.J. Kuo, unpublished observations).

Preincubation of HUVEC tubules with the MAPK kinase (MEK) inhibitor PD98056 strongly inhibited the ability of ES dimer to elicit cellular motility and MAPK activation in parallel (Fig. 7, C and D), indicating that the MAPK pathway is essential for the c18 motile response. In contrast, inhibition was not observed with SB203580, which inhibits the stress-activated p38 MAPK, indicating a selective requirement for the mitogenic MAPK pathway represented by MEK and MAPK, and not the stress response MAPK pathway represented by p38 and MKK3/4 (Fig. 7, C and D).

Inhibition of ES Oligomer-mediated Motility by Blockade of rac and cdc42

During NC1- and ES dimer-induced motility, rapidly demarginating cells appeared as early as 1–2 h with prominent lamellipodia, filopodia, and stress fibers (Fig. 2 A; see also videomicroscopy at Fig. 3 C and http://www.jcb.org./cgi/content/full/152/6/1233/DC1). Given the regulation of these structures by rho-family G proteins (Hall, 1998), we used *C. difficile* toxin B which glucosylates and inhibits rho-family GTPases. Preincubation of preformed HUVEC tubules with *C. difficile* toxin B produced complete blockade of ES dimer– and NC1-induced scatter (Fig. 8 A), with trypan blue staining confirming >95% cell viability (data not shown). To evaluate the potential role of individual rho-family GTPase family members, preformed HUVEC tubules were infected with adenoviruses encoding DN alleles of rac, rho, and cdc42 (Kalman et al., 1999). Strong inhibition of ES dimer–induced motility was observed with infection with racDN and cdc42DN viruses (Fig. 8 B). In contrast, no blockade was obtained with a rhoDN adenovirus, although cell rounding characteristic of stress fiber loss was observed (Fig. 8 B). Immunofluorescence revealed >95% infection of endothelial cells (Fig. 8 B, bottom). These results suggest that rac and cdc42 mediate the motile response to ES oligomers, and parallel findings that HGF-induced scatter is inhibited by racDN but not rhoDN (Ridley et al., 1995).

Autoregulatory Inhibition of c18 NC1-stimulated Motility by ES Monomer

Collagen XVIII undergoes proteolytic processing in vivo by cathepsin-L and elastase-like proteases (Wen et al., 1999; Felbor et al., 2000) to generate both NC1 trimers and ES domain monomers (Sasaki et al., 1998; Wen et al., 1999). Because of the clear oligomerization dependence of c18 motogenic activity, we investigated whether the physiologic cleavage product ES monomer might inhibit motility induced by the NC1 ES trimer. Although treatment of HUVECs or PC12 cells with NC1 or ES dimer resulted in characteristic cell motility, pretreatment with 40-fold molar excess of ES monomer before ES oligomer treatment strongly inhibited motogenic activity (Fig. 9, A–C). Identical inhibition of motility was also observed with human ES monomer produced in yeast or murine ES monomer produced in baculovirus (data not shown). In parallel, ES monomer pretreatment also abrogated c18 NC1- and ES

dimer–induced MAPK activation (Fig. 9 D) and tyrosine phosphorylation (LaMontagne, K.R., and C.J. Kuo, unpublished observations). These data suggest the possibility of negative autoregulation of endogenous c18 NC1 activity mediated by in vivo proteolytic release of monomeric ES domains and reiterate the requirement for ES domain oligomerization.

Discussion

An Evolutionarily Conserved ECM Collagen as Motogen and Morphogen

The ECM regulates cellular morphogenesis by serving as a passive structural support, a repository for growth factors, and a ligand for cellular receptors such as integrins and DDR tyrosine kinases (Giancotti and Ruoslahti, 1999; Vogel, 1999). The collagen XV/XVIII family has been particularly interesting because of a COOH-terminal ES domain not present in other collagens, and strong evolutionary conservation, being one of only two verterbrate basement membrane collagens conserved in *C. elegans* and *Drosophila* (Hutter et al., 2000; Hynes and Zhao, 2000). Despite this ancestral presence in invertebrate species, the physiologic functions of the XV/XVIII collagens have remained undefined.

In this work, we describe that the ES region of collagen XVIII functions as an effector domain regulating ECM-dependent motility and morphogenesis of numerous cell types. The composite nature of c18 in both vertebrates and *C. elegans*, with NH_2-terminal frizzled/wingless receptor (vertebrate) or unc-40/netrin receptor (*C. elegans*) domains, and COOH-terminal ES/motogen domains, could certainly facilitate sophisticated and modular control of cell migration by ECM during development and morphogenesis (Muragaki et al., 1995; Rehn et al., 1998; Ackley et al., 2001). The inactivity of the homologous c15 ES domain in dimeric and trimeric forms suggests a highly specific function for c18 (Fig. 4) and is consistent with structural differences between c15 and c18 NC1 domains (Sasaki et al., 2000), although certainly other contexts may be discovered in which c15 NC1 exhibits motogenic activity.

Motility Induced by Collagen XVIII NC1 Requires ES Domain Oligomerization

The c18 motogenic activity localizes to and absolutely requires oligomerization of the ES domain based on several lines of evidence. The COOH-terminal ES domain is obligately trimerized in c18 via intrinsically triple helical collagen structure and a redundant trimerization motif within NC1 (Muragaki et al., 1994; Rehn and Pihlajaniemi, 1994; Sasaki et al., 1998). The motogenic activity of the NC1 domain, representing an ES trimer, is reproduced by ES dimers in the form of either Fc-ES or the ES(Q7→C7) dimer. In turn, the activity of the ES(Q7→C7) dimer (IC_{50} 10 nM) contrasts starkly with the inactivity of monomeric ES domains which differ by essentially a single disulfide bond (inactive at 3,000 nM, the highest concentration tested). The stimulation of motility by the pure ES domain dimer but not ES monomers clearly demonstrates that ES oligomerization is both necessary and sufficient for motogenic activity (Fig. 3). This oligomerization requirement is further corroborated by the ability of NC1 and ES dimers,

but not ES monomers, to activate MAPK and tyrosine phosphorylation on ECM (Figs. 7 and 9; LaMontagne, K.R. and C.J. Kuo, unpublished observations).

Theoretically, oligomerization could facilitate c18 action upon specific cellular and/or matrix receptors. On the one hand, ES domain oligomerization could promote cross-linking of transmembrane signaling molecules, such as integrins, by analogy to growth factor receptors. On the other hand, NC1 ES trimers bind with 100–200-fold higher affinity than ES monomers to ECM components such as laminin/nidogen, perlecan, and sulfatides (Sasaki et al., 1998) and could regulate the association of matrix components with cellular receptors. We have observed ES domain binding to both membrane and matrix components, as has been described for FGF, VEGF, HGF, and Wingless (Yayon et al., 1991; Park et al., 1993; Reichsman et al., 1996; Binari et al., 1997; Chirgadze et al., 1999) and are actively investigating these two potential sites of action.

Comparison and Contrast between the c18 and HGF/MSP Motogens

As described here, collagen XVIII represents a novel motogen both structurally and functionally distinct from the archetypal HGF/MSP scatter factor class. The triple helical structure of collagen XVIII, with its three associated globular ES domains, little resembles the kringle structures of the plasminogen-like HGF and MSP proteins (Tamagnone and Comoglio, 1997). HGF induces motile scatter responses on plastic within 12 h but promotes tube formation and branching morphogenesis in collagen gels over a 4–6-d period (Tamagnone and Comoglio, 1997). In contrast, c18 NC1 does not promote disaggregation on plastic but induces rapid motility and disassembly on Matrigel as rapidly as 2 h, conditions in which HGF and MSP are inactive. Despite obvious differences in structure and context of activity, the motile responses induced by c18 and HGF exhibit similar downstream effector requirements for MAPK, rac, and cdc42 but not rho (Ridley et al., 1995; Potempa and Ridley, 1998; Figs. 7 and 8). The distinct activities of c18 and HGF suggest the potential to provide regulatory complexity during developmental morphogenesis and tissue remodeling.

Inhibition of ECM-induced Morphogenesis by the Collagen XVIII NC1 Domain

Culture of endothelial and nonendothelial cells on rich ECM preparations such as Matrigel is associated with pronounced alterations in cellular morphogenesis and adhesion. In the case of endothelial cells, these morphologic changes resemble the formation of vascular "tubes" with recapitulation of angiogenic processes including branching, migration, and differentiation (Folkman and Haudenschild, 1980; Grant et al., 1991). On the other hand, the significance of tube-like structures and aggregates formed by nonendothelial cell types such as fibroblasts and epithelial cells on ECM preparations is less clear. As described here, the c18 NC1 domain potently reverts ECM-induced morphologic changes of both endothelial and nonendothelial cell types, with cells assuming a morphology resembling cells plated on plastic. It thus seems likely that the c18 NC1 domain represents a previously unsuspected inhibitor

of ECM-induced morphogenesis. Additionally, the long-described aggregation and morphogenetic responses of cells to complex ECM preparations such as Matrigel could reflect a property which is not endothelial specific but rather represents a highly conserved, c18 NC1-sensitive response to ECM component(s) exhibited by diverse endothelial and nonendothelial cell types. These ECM components likely do not include collagen I or fibrin in isolation, since we have not observed effects of c18 NC1 against cells cultured on gels composed of these purified proteins (data not shown).

Negative Autoregulation by the Cleavage Product ES Monomer

The monomeric ES domain of c18 has been reported to be an antiangiogenic agent inhibiting endothelial cell proliferation and migration and inducing endothelial apoptosis in systems not using the ECM (O'Reilly et al., 1997; Dhanabal et al., 1999a,b; Yamaguchi et al., 1999; Dixelius et al., 2000). Here, we describe that the trimeric precursor c18 NC1 exhibits motility-inducing and morphogenic activities which are distinct from and in fact antagonized by the monomeric ES domain. Indeed, although c18 NC1 promotes motility, scatter, and MAPK induction, these activities are not exhibited by and are actually inhibited by ES monomer on ECM substrata (Fig. 9). This antagonistic relationship is potentially consistent with our inability to detect responses attributed to ES monomer such as inhibition of proliferation or apoptosis induction after c18 NC1 treatment (O'Reilly et al., 1997; Dhanabal et al., 1999a,b; Yamaguchi et al., 1999; Dixelius et al., 2000) (although these reports did not utilize ECM substrata) and reports in which ES monomers actually enhance rather than inhibit endothelial tube formation (Dixelius et al., 2000). Additionally, we have not observed significant antiangiogenic activity of c18 NC1 or ES dimers in corneal micropocket assays, in contrast to reports with ES monomers (Dixelius et al., 2000). It has been reported that both ES monomers and NC1 inhibit HUVEC migration in VEGF-based transwell chambers (although these assays did not employ matrix substrata, required endothelial cells of defined passage number, and required preincubation with factor [Yamaguchi et al., 1999]). Notably, an opposing relationship between ES monomer and trimeric c18 has been suggested based on purely structural considerations (Schuppan et al., 1998).

Both full-length c18 and fragments corresponding to NC1 and ES exist in both tissues and serum (Standker et al., 1997; Sasaki et al., 1998; John et al., 1999; Wen et al., 1999). Given the description of processing proteases which cleave monomeric ES from trimeric c18/NC1 (Wen et al., 1999; Felbor et al., 2000), the in vivo proteolytic cleavage of ES could constitute an autoregulatory feedback loop which terminates and actively opposes the activity of c18.

Evolutionarily Conserved Oligomerization-dependent Motility Regulation by the Collagen XVIII NC1/ES Domain

Among the twenty-odd vertebrate collagen genes, only collagen IV and collagen XV/XVIII are conserved within the *C. elegans* and *Drosophila* genomes (Prockop and

Kivirikko, 1995; Hutter et al., 2000), consistent with an evolutionarily conserved function of c18. Ackley et al. (2001) in this issue characterize the *C. elegans* collagen XVIII homologue *cle-1* which exhibits numerous structural similarities with vertebrate collagen XVIII, including conserved intron/exon boundaries, a triple helical collagenous domain, trimeric properties of the NC1 domain, and 45% amino acid identity in the ES domain. Notably, neuronal migration and axonal pathfinding deficits in *cle-1* mutants lacking the COOH-terminal ES domain are rescued only by trimeric CLE-1 NC1 and not by monomeric CLE-1 ES domains. Similarly, neuronal motility stimulated by wild-type trimeric CLE-1 is dominantly inhibited by overexpression of the physiologic cleavage product ES monomer but not by trimeric CLE-1 NC1, consistent with oligomerization dependence and negative autoregulation. The extensive structural and mechanistic parallels between *C. elegans cle-1* and vertebrate c18 suggest that collagen XVIII homologues throughout evolution regulate cell motility in a manner both dependent on ES domain oligomerization and negatively autoregulated by ES monomers. This convergence of function in organisms as diverse as vertebrates and *C. elegans* should provide a mechanistic foundation for elaborating the roles of c18 in motility-dependent processes such as angiogenesis, neurogenesis, branching morphogenesis, and metastasis in both vertebrate and invertebrate species.

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