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Regulation of Collagen Fibrillogenesis by Cell-surface Expression of Kinase Dead DDR2

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The assembly of collagen fibers, the major component of the extracellular matrix (ECM), governs a variety of physiological processes. Collagen fibrillogenesis is a tightly controlled process in which several factors, including collagen binding proteins, have a crucial role. Discoidin domain receptors (DDR1 and DDR2) are receptor tyrosine kinases that bind to and are phosphorylated upon collagen binding. The phosphorylation of DDRs is known to activate matrix metalloproteases, which in turn cleave the ECM. In our earlier studies, we established a novel mechanism of collagen regulation by DDRs; that is, the extracellular domain (ECD) of DDR2, when used as a purified, soluble protein, inhibits collagen fibrillogenesis *in-vitro*. To extend this novel observation, the current study investigates how the DDR2-ECD, when expressed as a membrane-anchored, cell-surface protein, affects collagen fibrillogenesis by cells. We generated a mouse osteoblast cell line that stably expresses a kinase-deficient form of DDR2, termed DDR2/-KD, on its cell surface. Transmission electron microscopy, fluorescence microscopy, and hydroxyproline assays demonstrated that the expression of DDR2/-KD reduced the rate and abundance of collagen deposition and induced significant morphological changes in the resulting fibers. Taken together, our observations extend the functional roles that DDR2 and possibly other membrane-anchored, collagen-binding proteins can play in the regulation of cell adhesion, migration, proliferation and in the remodeling of the extracellular matrix.

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Introduction

Collagen type I in its mature fibrillar state is the major component of the extracellular matrix (ECM) in most mammalian tissues.[1] Collagen fibers impart mechanical strength to the tissue and interact with cells through cell surface receptors and soluble proteins, which is integral to cell proliferation, migration, survival, attachment and cellular differentiation. The assembly of collagen fibers (fibrillogenesis) is a complex process regulated, in part, by a variety of collagen-binding proteins and other molecules that may directly or indirectly interact with the collagen molecules and fibrils. Several collagen-binding proteins such as decorin[2], lumican[2], cartilage oligomeric matrix protein,[3] fibromodulin,[4] SPARC,[5] and matrilin,[6] etc. have been shown to influence collagen fibrillogenesis. However, almost all these proteins occur as cell-secreted, soluble proteins in the ECM. It is not well understood to what extent collagen-binding proteins anchored to the cell surface affect the assembly of collagen fibrils in the ECM.

The collagen-binding membrane proteins, discoidin domain receptors 1 and 2 (DDR1 and DDR2) belong to the family of receptor tyrosine kinase and

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Abbreviations used: ECM, extracellular matrix; DDR, discoidin domain receptor; ECD, extracellular domain; KD, kinase dead; MMP, matrix metalloprotease; TEM, transmission electron microscopy; HP, hydroxyproline; vWF, von Willebrand factor; IP, immunoprecipitation.

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are expressed in a variety of mammalian cells.[7,8] These transmembrane glycoproteins (~125 kDa) have been found to be over-expressed or atypically expressed in several malignancies,[9–12] and regulated in diseases such as atherosclerosis,[13] lymphangioleiomyomatosis,[14] rheumatoid arthritis,[15] and osteoarthritis.[16] DDRs are characterized by three distinct regions:[17] an extracellular domain (ECD), which is responsible for collagen binding, a transmembrane region and an intracellular kinase domain. Binding of collagen(s) to the DDR ECD is known to induce tyrosine phosphorylation of the DDR kinase domain;[7,8] prolonged activation of the DDR kinase domain results in upregulation or activation of matrix metalloproteases (MMPs 1, 2, 9 and 13), which cleave and degrade the collagen fibers in the ECM.[7,14,16]

A second mode of collagen regulation reported earlier by our laboratory shows that the ECD of DDR1 or DDR2 when expressed as a soluble protein can modulate fibrillogenesis of collagen type 1 *in-vitro*.[18,19] In particular, we found that DDR2 ECD delays collagen fibrillogenesis and the collagen fibers formed in the presence of DDR2 ECD were thinner and lacked the native D-periodic banded structure. However, these earlier observations were based mainly on using purified collagen and a soluble form of DDR2 ECD, whereas thus far the DDR2 ECD has only been reported as an integral component of the membrane-anchored, full-length DDR2 receptor.

Therefore, in this study we asked if the expression of DDR2 ECD anchored to the cell surface preserves the capacity to modulate collagen fibrillogenesis for collagen endogenously secreted by the cells. To address this question, we created stably transfected mouse osteoblast cell lines to express a DDR2 isoform, named DDR2/-kinase dead (KD), which resembles the naturally occurring full-length DDR2 except that it lacks the kinase domain. We could thus ensure that our observed effects on collagen morphology and structure would be due only to DDR2 ECD interaction and not through the cleaving action of MMPs, known to be activated upon DDR2 kinase domain activation. Since mouse osteoblasts endogenously secrete collagen, we were able to examine the effects of DDR2/-KD expression on collagen morphology and deposition in the ECM by using techniques such as transmission electron microscopy (TEM) and hydroxyproline (HP) assay. We elucidate how the cell-surface expression of DDR2 ECD plays a major role in regulating the rate of collagen fibrillogenesis and morphology of collagen fibers. Our results demonstrate a novel mechanism of collagen regulation by DDRs and signify the importance of cell-surface-anchored, collagen-binding proteins in regulating collagen fibrillogenesis.

Results

Characterization of DDR2/-KD and stable cell lines

To evaluate the changes in ECM induced by expression of DDR2/-KD, we utilized mouse osteoblasts cells (MC3T3, E1 subgroup-4 clone), which are known to endogenously secrete collagen and generate well defined collagen fibers in their ECM. These cells were used previously to demonstrate that overexpression of lysyl hydroxylase-2b leads to defective collagen fibrillogenesis.[20] Collagen assembly in the ECM of these cells takes one to several weeks; therefore, it was necessary to stably transfect these cells with DDR2/-KD to observe its effect on collagen fibrillogenesis.

Figure 1a shows a representation of the DDR2/-KD construct, which leads to the expression of a truncated DDR2 protein, preserving the extracellular, transmembrane and juxtamembrane regions but

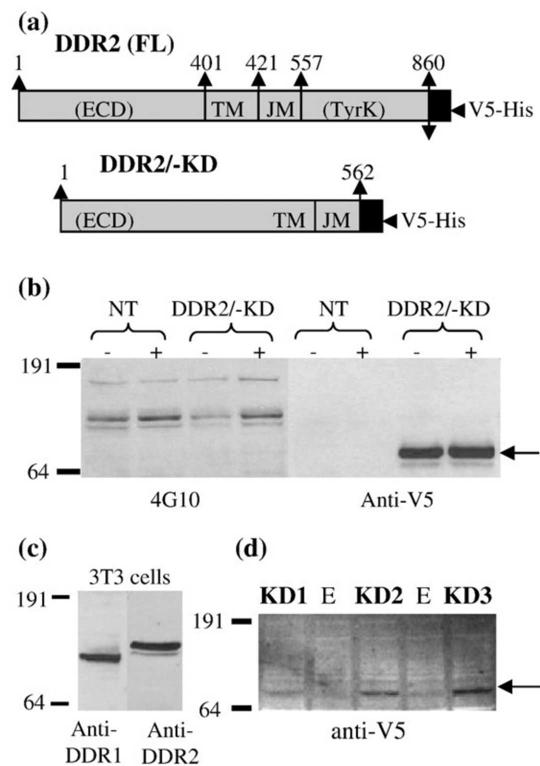

Fig. 1. Creation of stable cell lines expressing the recombinant protein DDR2/-KD. (a) A schematic representation of V5 His-tagged full-length mouse DDR2 and of the V5 His-tagged, membrane-anchored, kinase-deficient DDR2 (DDR2/-KD) transmembrane protein. The extracellular (ECD), transmembrane (TM), juxtamembrane (JM) and tyrosine kinase (TyrK) domains are indicated. The numbers denote the sequence of amino acids in our recombinant proteins. (b) DDR2/-KD does not undergo collagen-induced tyrosine phosphorylation. Following SDS-PAGE, Western blot of whole cell lysates from native (NT) or transiently transfected (DDR2/-KD) HEK293 cells before (–) and after (+) collagen stimulation, were probed using anti-phosphotyrosine (4G10) or anti-V5 antibodies. While the ~125 kDa band indicates phosphorylation of endogenously occurring DDRs, no phosphorylation signal was present for DDR2/-KD (indicated by an arrow). (c) Western blot indicating expression of endogenous DDR1 and DDR2 in 3T3 cells. (d) Three stable MC3T3 cell lines, KD1, KD2, and KD3, were selected for our study, which show increasing levels of DDR2/-KD expression (arrow). E, Empty lane.

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