ROLE OF OLIGOMERIZATION IN DISCOIDIN DOMAIN RECEPTORS – COLLAGEN TYPE I INTERACTION

DISSERTATION

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ABSTRACT

Discoidin domain receptors (DDR1 and DDR2) are unique tyrosine kinase receptors (RTK) in that they bind to and are activated by collagens, including collagen type I. The mechanism(s) of DDRs binding to collagen is not completely understood. It is generally accepted that for receptors from the RTK family, ligand binding induces a transition from the monomeric to oligomeric state; however, the oligomeric state of DDRs and its modulation by collagens is not yet characterized.

Using the purified DDR extracellular domain (ECD) we have been able to quantitatively describe its binding to collagen type I. We found that both DDR1 and DDR2 ECD require oligomerization for binding to collagen type I. At the same time we found that DDRs can clearly distinguish between the monomeric and fibrillar states of collagen with an increased affinity toward monomeric collagen. This can have significant physiological relevance, as the implication is that DDRs signaling is inhibited in the presence of mature collagen fibers.

Using fluorescently labeled DDR1 and live cell imaging, we have found that collagen induces rapid aggregation and internalization of DDR1,
followed by its incorporation in the early endosome. Fluorescence resonant energy transfer (FRET) experiments in live cells, confirmed that DDR1 exists as a dimer prior to collagen stimulation and that collagen-induced internalization applies preferentially to the dimeric fraction of the receptor population. The dynamics of receptor internalization suggest that DDR1 dimerization, collagen binding and receptor phosphorylation are independent events, separated both temporally and spatially. Further, in vitro work with purified DDR ECD, and live cell work with recombinant DNA, demonstrated a novel mechanism of collagen regulation by DDRs, namely that binding of DDRs to collagen can directly modulate collagen fibrillogenesis. Taken together, our studies improved our understanding of DDR-collagen interaction and generated new evidence that supports the role of DDR receptors as potent regulators of collagen deposition in the extracellular matrix.
Dedicated to

My wife, Gina
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PUBLICATIONS


Parts of the work presented in this dissertation were included in the above publications as follows:

**Chapter II**

The data from Sections 2.2., 2.3., (2.3.1., 2.3.2.) and 2.4., was included in the above publications. I restructured and rewrote the text for inclusion in this dissertation. The SEC experiments were done with the help of Dr. Lawrence Druhan.

**Chapter III**

Chapter III was submitted for publication as it is, except for the Sections 3.1. and 3.4., that I rewrote for inclusion in this dissertation. Dr. Terry Elton and Dr. Maqsood Chotani helped me in designing and creating the DDR-GFP fusion proteins. The manuscript was edited by Dr. Gunjan Agarwal and the other coauthors.

**Chapter IV**

Most of Chapter IV results were published in the above publications. I restructured and rewrote the text for inclusion in this dissertation. Parts of the AFM experiments were done by Daniel F. Iscrue and the TEM imaging and image analysis was done by Dr. Gunjan Agarwal.
FIELDS OF STUDY

Major Field: Biophysics
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CHAPTER 1

COLLAGEN TYPE I, DISCOIDIN DOMAIN RECEPTORS OVERVIEW

1.1. INTRODUCTION

Cells of multicellular organisms are surrounded by an intricate network of macromolecules, known as the Extracellular Matrix (ECM). Besides acting as a scaffold to stabilize the physical structure of tissues, ECM has an active and complex role in regulating the behavior of the cells that contact it, influencing their survival, development, migration, proliferation, shape, and function (Alberts 2007). Two main classes of macromolecules make up the ECM: (1) polysaccharide chains of the class called glycosaminoglycans (GAGs), which are usually found covalently-linked to proteins in the form of proteoglycans, and (2) fibrous proteins, including collagen, elastin, fibronectin, and laminin, which have both structural and adhesive functions. Of all these macromolecules, collagen is the most abundant ECM protein.

1.2. COLLAGEN TYPE I

Collagen constitutes a large protein family of more than 20
members. Within this family, the fibrillar collagens (types I, II, III, V and XI) play a key architectural role (Prockop 1995). Collagens play a pivotal role in regulating cellular differentiation and pattern formation during embryogenesis and postnatal development. Increased synthesis of fibrillar collagens or perturbed turnover correlates with a variety of human diseases including fibrosis, vascular disease and tumor angiogenesis (Myllyharju 2001). On the other hand, enhanced degradation of the collagen matrix is a prelude to cancer metastasis.

Collagen fibers play a major role in defining ECM mechanical properties, which have significant physiological relevance in vasculature, skin and lungs where it undergoes elastic extension and in articular cartilage where it undergoes elastic compression (Brown 1994). At the molecular level, collagen fibers are subjected to mechanical stresses by cells through cell surface proteins (Lee 1999). Also, it was reported that cellular responses are mediated by the stiffness of the matrix in which they move (Freyman 2002).

Of all collagen types, the most abundant is collagen type I, which is found throughout the body except in cartilaginous tissues. It is the most widely studied collagen and often serves as a model to understand interactions and properties of other collagen types, many of which bear structural similarities with collagen type I.

1.2.1. Collagen biosynthesis and fibrillogenesis

Collagen type I is composed of three polypeptide chains, commonly known as α-chains, which assemble together to form the collagen triple helix
– in the case of collagen type I – two $\alpha1(I)$ chains and one $\alpha2(I)$ chain (Kadler 1995). The $\alpha$-chains are comprised of a Gly-X-Y triplet repeat, where X and Y can be any amino acid but are frequently proline and hydroxyproline. The presence of glycine at every third residue is a prerequisite for folding of the three $\alpha$-chains into a triple helix.

The biosynthesis of collagen has traditionally been considered to occur in two stages. The first stage includes the intracellular steps that produce a folded, triple-helical, soluble procollagen molecule that is secreted from the cell. As the $\alpha$-chains are synthesized they undergo cotranslational hydroxylation of the proline and lysine residues. The nascent $\alpha$-chains undergo glycosylation at the hydroxylysyl residues. Hydroxylation of peptidyl proline induces conformational changes in the peptide backbone that promotes triple helix formation. It is understood that assembly of the pro-$\alpha$-chains into procollagen, starts after association of the C-propeptides from the three $\alpha$-chains and that the triple helix folding propagates in a C to N direction in a zipper-like action. Formation of the triple helix prevents further hydroxylation of the procollagen molecule; its condensed structure conferring high stability and making it resistant to cleavage by proteases such as pepsin, trypsin or chymotrypsin but not collagenase.

The second stage of collagen synthesis includes extracellular steps of proteolytic conversion of procollagen to collagen and its polymerization into fibrils, a process known as fibrillogenesis (Figure 1.1.). The newly generated procollagen molecules are excreted from the cell into ECM, and undergo cleavage of the N and C propeptides by the procollagen N and C proteinases.
Figure 1.1. Collagen biosynthesis occurring in cell-surface crypts. Collagen is secreted by cells as procollagen consisting of a 300-nm-long triple-helical domain (comprised of three α-chains each of ~1000 residues) flanked by a trimeric globular C-domain (the right-hand side of the diagram) and a trimeric N-propeptide domain (the left-hand side of the diagram). Upon secretion, procollagen undergoes cleavage of the N- and C-propeptides by procollagen proteinases specific to these domains. The triple-helical collagen molecules then spontaneously self-assemble into cross-striated fibrils which are stabilized by covalent cross-linking, initiated by oxidative de-amination of specific lysine and hydroxylysine residues in collagen by lysyl oxidase (Kadler, 1996).
The resulting collagen molecule is rod-like (300nm x 1.5 nm) and spontaneously self assembles to form fibrils. In literature, it is common to denote the early collagen polymers as fibrils and the later, longer, thicker polymers as fibers. The process of collagen fibrillogenesis is thought to be entropic, the major driving force being the hydrophobic interaction between collagen molecules leading to minimization of the surface exposed to water. Collagen fibers, that are formed in vitro or in vivo are characterized by a specific organization of collagen molecules - staggered by integrals of D with respect to one another, where D ~ 67 nm (Kadler 1996). Given the length of the collagen monomers – 300 nm, they can stagger in five possible configurations starting from full overlap (0 D stagger) to only one overlap (4 D stagger). All of the above combinations are found during fibrillogenesis, with a predominance of 0 and 1 D stagger during the latter stages of the fiber development, while the higher stagger patterns 2, 3 and 4 D were encountered in the early stage of fiber formation (Figure 1.2.). Also, the fiber growth is surface-diffusion limited, with the incoming monomers – or small fibrils – interacting with the molecules from the outer layer of the fiber (Parkinson, 1994).

The new monomers preferentially accrete to the monomers at the fiber tip resulting in an abundance of the 4 D staggers. However, due to the entropic nature of the process, the collagen molecules further minimize their surface area by contact with other collagen molecules. This induces rearrangements in the fiber organization with the end effect that 0
and 1 D staggers will predominate and give the observed D periodicity of the mature fiber. The fibers are further stabilized \textit{in vivo} by lysyl oxidase cross-linking between the collagen molecules.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{collagen_molecules_assembly.png}
\caption{Schematic representation of collagen molecules assembly. The collagen molecule is approximately 4.5 D-periods in length. Electron microscopy has demonstrated that collagen molecules are staggered by integrals of D with respect to each other. Therefore there are five specific ways in which two molecules can assemble wrt. each other, defined as the 0 D, 1 D, 2 D, 3 D and 4 D staggers, depending upon the length of the overhang. (Parkinson, 1994).}
\end{figure}

\textbf{1.2.2. Degradation of collagen fibers}

Collagen degradation takes place during a variety of physiological and pathological tissue-remodeling processes, including development, tissue repair, degenerative diseases of the connective tissue and cancer. There are three well defined molecular pathways of collagen degradation. The best understood involves a group of secreted or membrane-associated
matrix metalloproteinases (MMP) that directly cleave collagens within the perinuclear or extracellular environment (Birkedal-Hansen, 1987). MMP1 (collagenase) cleaves the three α-chains of type I, II, and III collagen. The generated fragments have a decreased thermal stability and become substrates for MMP2 and MMP9 (gelatinases), which further degrade the fragments (Kadler 1995). A second degradation pathway is mediated by cathepsin K and is specific for osteoclast mediated bone resorption (Gelb 1996). The third collagen degradation pathway takes place intracellularly, and involves the binding of collagen fibers to specific cell-surface receptors (integrins and Endo 180 mannose family receptor), followed by intracellular uptake, lysosomal delivery and proteolytic degradation by cathepsins (East 2002).

1.2.3. Regulation of collagen by collagen-binding proteins

A variety of cellular proteins, both soluble and membrane anchored are reported to interact with collagen, and in response, regulate collagen synthesis, fibrillogenesis and degradation. Currently there are several soluble proteins present in the ECM and five known transmembrane collagen receptors that interact directly with the collagen triple helix:

**Extracellular proteins**

A number of soluble ECM proteins are known to interact with collagen. Among them are small leucine-rich proteoglycans such as fibromodulin, lumican and decorin, fibronectin (a large integrin-associated
proteoglycan), matrilin and periostin. Several of these proteins were shown to regulate collagen fibrillogenesis and/or the assembly of the collagen fiber network (Kalamajski 2007, Sini 1997, Carlson 2003, Norris 2007).

Transmembrane Proteins

**Integrins** form a large family of receptors that mediate cell adhesion to the ECM. Eight β-and eighteen α-subunits combine to form twenty four distinct integrins (Hynes 2002). Both subunits have a large modular extracellular domain, followed by a single transmembrane helix and a short cytoplasmic domain. Four integrin heterodimers are known to bind to collagen (α₁β₁, α₂β₁, α₁₀β₁, α₁₁β₁). While the mechanism of integrin binding to collagen is well understood, much less is known regarding signaling mechanisms. Interaction of collagen with integrins is understood to have important biological functions including regulation of collagen synthesis and upregulation of MMPs that cleave the collagen matrix. Soluble α₁ and α₂ domains were shown to inhibit collagen fibrillogenesis *in vitro* (Jokinen 2004). Also collagen binding to integrins is reported to influence fibroblast proliferation, platelet adhesion, keratinocyte adhesion and growth plate morphogenesis and function.

**Glycoprotein VI (GPVI)** is a collagen receptor found on platelets that plays a central role in the formation of a hemostatic plug at sites of vascular injury (Ruggeri 2002). GPVI consists of two immunoglobulin-like (IG) domains, a heavily O-glycosylated mucin-like stalk region, a transmembrane helix and a
short cytoplasmic domain containing binding motifs for calmodulin and SH3 domains (Moroi 2004). The functional receptor is a stable complex of GPVI with the Fc receptor γ chain, which contains in its cytoplasmic tail an immunoreceptor tyrosine activation motif (ITAM). Binding of fibril forming collagens to the GPVI ectodomain results in ITAM phosphorylation by the Src family tyrosine kinases which are thought to be constitutively associated with GPVI through their SH3 domains. The signaling cascade downstream leads to platelet’s activation.

**Leukocyte-associated IG-like receptor 1 (LAIR-1)** delivers inhibitory signals to various cells of the immune system (Meyaard 1997). LAIR-1 structure consists of a single IG domain, a transmembrane helix and a short cytoplasmic domain containing two immunoreceptor tyrosine based inhibitory motifs. Collagen type I and collagen type III were found to act as functional inhibitory LAIR-1 ligands (Lebbink 2006).

**The mannose receptor family** is comprised of four members that share common a domain architecture: mannose receptor (MR), the M-type phospholipase A2 receptor (PLA2R), DEC-205, and Endo180 (also known as uPARAP). The extracellular domains of these proteins consist of an N-terminal cystein-rich domain, a single fibronectin type 2 (F2) domain, and eight C-type lectin-like domains (CTLDs) (East 2002). The short cytoplasmic domains contain functional endocytosis signals. All of those receptors have shown binding affinity toward fibril forming collagens, collagen IV, as well as gelatin, and three
of these - PLA2R, MR and Endo 180, were reported to mediate collagen endocytosis.

The latest addition to the class of transmembrane collagen binding proteins is the Discoidin Domain Receptor family, discussed in detail in the following section.

1.3. DISCOIDIN DOMAIN RECEPTORS (DDRS)

Discoidin domain receptors (DDR1 and DDR2) are members of the receptor tyrosine kinase (RTK) family, which bind to and are activated by triple helical collagens (Vogel 2006).

1.3.1. Expression of DDRs

DDRs are widely expressed in normal human tissues (Vogel 1999, Franco 2002), with DDR1 found mainly in epithelial cells (Alvez 1995) but also in leukocytes (Matsuyama 2005), and DDR2 mainly expressed in mesenchymal cells (Alvez 1995). DDRs are expressed early in embryonic development and are found in many adult tissues, with high levels of DDR1 found in lung, kidney, breast and brain tissue, while DDR2 is found predominantly in skeletal muscle, skin, kidney and lung tissue (Vogel 2006).

1.3.2. Structure of DDRs

Both DDR1 and DDR2 are composed of an N-terminal ~150 amino acid discoidin homology (DS) domain, followed by a “stalk” region, a
sequence of ~ 220 amino acids unique to DDRs, a transmembrane (TM) domain, a large cytosolic juxtamembrane domain and a C-terminal catalytic tyrosine kinase domain. DDR1 and DDR2 show 58% homology in the discoidin domain region, and only 44% homology in the stalk region (Abdulhussein 2004). While DDRs are the only RTKs containing the DS domain, more than a dozen other mammalian transmembrane and secreted proteins are known to incorporate it in their sequences (Figure 1.3.).

The crystal structure of the DDR2 DS domain was recently resolved by NMR spectroscopy (Ichikawa 2007). The overall fold was found to consist of eight major β-strands (1-8), which are arranged in two antiparallel β-sheets of five (1, 2, 7, 4, 5) and three (8, 3, 6) strands packed against each other (Figure 1.4.). The resulting β-barrel structure is common to other DS domains: the coagulation factors V and VIII C2 domains and the neurophilin-1 B1 domain. At the top of the β-barrel core protrude six juxtaposed loops (L1-L6), among which L1, L2 and L4 were found to be different from those of other DS domains in their length and conformation. The bottom of the β-barrel core is closed by three interconnecting straight segments, and the N-and C-termini are connected with a disulfide bond between the Cys30 and Cys185 residues.
Figure 1.3. The family of proteins with DS domains. Abbreviations are as follows: F-A, A-domain in Factor V and VIII; CBP, carboxypeptidase; EGF, epidermal growth factor; TK, tyrosine kinase; A5, homology to A5 antigen; Ig, immunoglobulin; LamG, laminin-G; FIB, fibronectin-like; CUB, complement binding; and MAM, meprin/A5/PTPmu. Tyrosines in the N-P-X-Y motives of GCTK and DDR1b are highlighted. Proteolytic processing of DDR1 is indicated (Vogel, 1999).

Transferred cross-saturation measurements (TCS) on the DDR2 DS domain structure showed that collagen binds to the loop region, where the L1, L2, L3, L4 and L6 loops form a trench suitable to accommodate the triple helix of the collagen fiber.
The structure of the DDR1 DS domain is not yet known, however, utilizing the known structures of coagulation factors V and VIII, a molecular model of this domain was generated (Leitinger 2003, Abdulhussein 2004). Two loops, L1 and L3, were found to be essential for collagen binding and receptor activation.

Figure 1.4. Ribbon diagram of the DDR2 DS domain. L1–L6 refers to loops 1–6. The disulfide bonds between Cys30 and Cys185, and between Cys73 and Cys177 are shown as stick models colored yellow (Ichikawa, 2007).
1.3.3. Isoforms of DDRs

Compared with most other RTKs, the juxtamembrane regions of DDR1 and DDR2 are much longer (176 and 147 aa, respectively). In several cell lines DDR1 is partially processed into a 62 kDa membrane anchored β-subunit and a 54 kDa soluble ECD-containing α-subunit (Hou 2002). This process is significantly enhanced upon prolonged DDR1 activation. Five isoforms of DDR1 have been identified, all generated by alternative splicing in the cytoplasmic region (Alves 2001). The longest isoform is DDR1c that contains 919 amino acids. DDR1a isoform lacks 37 amino acids in the juxtamembrane domain and 6 amino acids in the kinase domain, whereas DDR1b isoform lacks 37 amino acids in the juxtamembrane domain (Figure 1.5.). DDR1b is the predominant isoform during embryogenesis, whereas DDR1a is commonly found in several human mammary carcinoma cell lines (Perez 1994). DDR1d and DDR1e are truncated variants that lack either the entire kinase region or parts of the juxtamembrane region and the ATP binding site.

Although the splice variants for DDR2 are not yet characterized there is evidence to support that they do exist. Several protein species for DDR2 have been detected in cultured human smooth muscle cells at various molecular weights: 130, 90, 50 and 45 kDa, along with two transcripts at 9.5 and 4.5 kb (Ferri 2004). Independent studies have identified multiple transcripts for DDR2 in both cancerous and normal cell lines (Alves 1995, Karn 1993, Lai 1994).
Figure 1.5. Schematic representation of the different isoforms of DDR1. The upper panel shows the organization of the genomic locus of DDR1 between exon 9 and 14. The various splicing events that result in the previously known isoforms DDR1a, DDR1b, and DDR1c, as well in the novel isoforms DDR1d and DDR1e, are depicted underneath the genomic locus. In the lower panel, the predicted length, molecular weight, and overall structure of the different DDR1 isoforms are shown (Alves, 2001).
**1.3.4. DDR-collagen interaction**

While both DDRs bind to the native triple-helical structure of fibrillar collagens (types I-III and V), DDR1 binds also to non-fibrillar collagen type IV, while DDR2 binds to the non-fibrillar collagen type X (Leitinger 2006). However, the mechanism by which DDR ECD binds to collagen is not completely characterized. It is reported that DDR2 binds at three sites on the collagen type I molecule (Agarwal 2002). A recent study revealed that the DDR2 binding site is confined to the D2 domain of collagen type II, and that the amino acid sequence GARGQAGVMGFO corresponding to amino acids 394-405 has the highest binding affinity for DDR2 (Konitsiotis 2008, Leitinger 2004). Much less is known about DDR1 interaction sites on collagen (Abdulhussein 2004).

The key features understood about interaction of DDRs with collagen are: (i) the entire ECD of DDR1 is necessary while only the DS domain of DDR2 is sufficient for collagen binding, (ii) DDRs require dimerization (or oligomerization) for interacting with collagen and (iii) activation of DDRs by collagen is slow (hours rather than seconds) compared to activation of typical RTKs by their ligands (Shrivastava 1997).

Not much is known about the specifics of DDRs activation mechanism, and about the biomolecular interactions involved in the downstream signaling pathway. Mutational analysis of DDR1 binding to collagen (Curat 2001), showed that while epitopes present on the discoidin domain affect both the binding of the receptor to collagen and its activation, the effects are not always directly correlated. Mutation of the residue Pro 158 decreased the
collagen-binding affinity with no effect on receptor phosphorylation. Mutations on
two residues Arg 179 and Glu 181, were shown to increase collagen-binding
affinity and at the same time decrease receptor phosphorylation. A recent study
(Noordeen 2006) demonstrated that a leucine-zipper motif in the transmembrane
domain of DDR1 is critical for receptor activation, but not for receptor
dimerization.

1.3.5. Role of DDR oligomerization

Several groups (Alves 2001, Leitinger 2003, Abdulhussein 2004,
Vogel 2006) have tried to understand the role of DDR oligomerization in collagen
binding, often with contradictory results. It is evident that the DDR ECD is most
important in its interaction with collagen. This ECD is found not only in the full-
length DDR but also in the kinase dead isoforms of DDR1 (Alves 2001) and as a
have reported that GST tagged DDR1 ECD (GST-DDR1) is detected as a 73 kDa
protein (likely a monomer) and binds directly to immobilized collagen type I using
ELISA type binding assays. On the other hand monomeric, histidine tagged ECD
of DDR1 (His-DDR1) described by Leitinger (2003) failed to show a binding
response to immobilized collagen when examined using ELISA.

It was also recently reported that full-length DDR1 exists as a dimer
on the cell surface (Noordeen 2006) and that collagen binding does not increase
DDR1 dimerization on the cell surface as ascertained using Western blotting.
The same study reported that for the full-length receptor expressed on the
cell membrane, no single domain is solely responsible for dimerization but that multiple interactions along the entire DDR1 molecule collaborate in dimer formation. It is still not known whether higher oligomeric forms of DDRs are present on the cell surface or as shedded proteins, and whether they play a role on collagen binding and subsequent receptor activation.

1.3.6. DDRs activation and signaling

The catalytic domains of DDRs have a total of 15 tyrosine residues in the case of DDR1 and 13 tyrosine residues in the case of DDR2, that are potential phosphorylation sites following activation by collagen. It was shown that tyrosine 513 in DDR1b directly associates with the PTB domain of ShcA upon receptor activation (Vogel 1997). In macrophages, phosphorylation of ShcA by DDR1 led to activation of the TRAF6 complex, which triggers the p38 mitogen-activated protein kinase and NFκB pathways (Matsuyama 2003).

Other phosphotyrosine binding molecules were found to directly interact with DDR1 (Figure 1.6.), among them Shp-2 and SH2 domains containing tyrosine phosphatases, Nck2, SH2 and SH3 domains containing adapter proteins (Vogel 1993). DDR1b contains the motif LLXNPXY that associates with the phosphotyrosine binding domain of the ShcA adapter protein upon collagen induced tyrosine phosphorylation (Vogel 1997). The juxtamembrane region of the DDR1a binds to fibroblast-growth factor receptor 2 and triggers the migration and pseudopod extension of leukocytes (Foehr 2000, Kamohara 2001).
Figure 1.6. DDRs signaling network. Schematic representation of signaling molecules downstream of DDR1 and DDR2. Red arrows indicate a direct binding, while gray arrows show an indirect interaction (Vogel 2006).
Also tyrosine 881 was shown to mediate the binding of DDR1 to the p58 subunit of phosphatidyl-inositol-3 kinase (L'Hote 2002). Full activation of DDR2 requires the presence of ShcA and Src-like tyrosine kinase (Ikeda 2002). ShcA binds to the juxtamembrane region of DDR2, the interaction being mediated by the SH2 domain of the ShcA (Yang 2005). In human breast cancer cells, it was suggested that DDR1 receives lateral input from other transmembrane receptor/ligand complexes, such as Frizzled and Wnt5a (Dejmek 2003).

It is reported that prolonged activation of both DDR1 and DDR2 upregulates matrix metalloproteinases (MMPs 1, 2 and 9), which in turn cleave the ECM (Ferri 2004, Hou 2002). Activation of DDR1 is also known to upregulate the expression of collagen types I and III α-chains and of integrin α2. DDR1 mediates the p53 induced Ras/Raf/MAPK signaling cascade through direct interaction with the Ras effector protein (Ongusaha 2003). At the same time recruitment and activation of plasma membrane associated PI3 kinase by phosphorylated DDR1 it is believed to increase breast cancer tumor’s adhesion and minimize tumor proliferation (Dejmek 2003).

1.3.7. Physiological Role(s) of DDRs

Normal tissue

Both DDR1 and DDR2 are expressed early during embryonic development and in normal adult tissue. Targeted deletion of either DDR1 or DDR2 genes revealed valuable insights in the physiological functions
of the two receptors. Mice lacking DDR1 are viable but exhibit dwarfism, delayed development of mammary glands and proteinurea, which is caused by swelling of the glomerural basement membrane (Vogel 2001). Vessel injury of DDR1-null mice, showed this receptor to be involved in vascular smooth cell adhesion, proliferation and MMP production (Hou 2001, 2002). Overexpression of a dominant negative form of DDR1 in cerebellar cell and organ cultures caused a strong reduction of neurite outgrowth, indicating an essential role in tissue development (Bhatt 2000). Overexpression of DDR1a was found to promote cell adhesion and at the same time trigger the migration and pseudopod extension of leukocytes (Foehr 2000, Matsuyama 2003). Cells isolated from DDR1 knockout animals exhibited reduced migration rates as compared with wild type cells (Hou 2001, Hou 2002, Olaso 2002). These results indicate that DDR1 plays an important role in regulating cell morphogenesis, differentiation and proliferation in several organs, including the mammary gland, the vasculature and the kidney (Vogel 2006).

Mice lacking DDR2 exhibited dwarfism, caused by a reduced proliferation rate of chondrocytes (Labrador 2001). Skin fibroblasts from DDR2-null mice showed reduced migration rates, which results in delayed healing of epidermal wounds.

**Disease**

DDR1 is overexpressed in several malignancies such as breast, brain, colon, ovaries, lungs and esophagus (Yamanaka 2006, Heinzelmann-
Schwarz 2004, Weiner 2000, Nemoto 1997, Perez 1996). While DDR1 mRNA was found to be upregulated in the malignant cells, DDR2 was present in the stromal cells surrounding the malignant cells (Alves 1995). DDR1 has been demonstrated to be a direct transcriptional target of the p53 tumor-suppressor gene, suggesting it plays a role in carcinogenesis.

Both DDR1 and DDR2 play a crucial role in a variety of human diseases such as pulmonary fibrosis (Matsuyama 2006), atherosclerosis (Franco 2002, Ferri 2004) and arthritis (Balduino 2005), while DDR1 alone is involved in pituitary adenoma (Yoshida 2007), congestive heart failure (Andersson 2006), and lymphangioleiomyomatosis (Ferri 2004).

The existing data indicate that DDR receptors play an important role in both human health and disease. Further research into elucidating the mechanisms by which DDRs exert their function can result in development of new therapeutic strategies.
1.4. THESIS AIMS

The focus of this work is to achieve a better understanding of DDR-collagen interaction and its physiological significance. Toward this end we propose to test the following hypotheses:

- The collagen fibrillar state defines the properties of the extracellular matrix and the cell-matrix interactions. **Our hypothesis is:** collagen binding to the DDR receptors is mediated by the fibrillar state of collagen, thus the DDRs collagen-binding affinity is different for monomeric vs. fibrillar collagen. To test this we propose to obtain a quantitative characterization of DDRs binding to collagen type I, by determining the kinetic association and dissociation rates using surface plasmon resonance.

- DDR1 oligomerization was shown to be necessary for **in vitro** binding to collagen. **Our hypothesis is:** the oligomeric state of DDR1 on the plasma membrane mediates its interaction with collagen. To test this we propose to use Fluorescent Resonant Energy Transfer (FRET) microscopy that will allow mapping of the DDR1 oligomeric state and distribution on live cells.

- Collagen-binding proteins, both soluble and membrane anchored were shown to modulate collagen fibrillogenesis. **Our hypothesis is:** collagen receptors DDR1 and DDR2 have an impact on both the kinetics of fibrillogenesis and the characteristics of collagen fibers. We propose to test this using both **in vitro** and cell-based assays.
CHAPTER 2

COLLAGEN TYPE I BINDING TO DDR1 AND DDR2 IS MEDIATED BY THE FIBRILLAR STATE OF COLLAGEN

2.1. INTRODUCTION

Background

The Extracellular Matrix (ECM) acts as a scaffold to stabilize the physical structure of tissues, and at the same time exerts an active and complex role in regulating the behavior of the cells that contact it, influencing their survival, development, migration, proliferation, shape, and function (Alberts 2007). As a major component of the ECM, collagen type I mediates cell-matrix interactions (Kadler 1995). Fibrillar states of collagen define the mechanical properties of ECM, which have been demonstrated to directly influence cell migration and proliferation (Freyman 2002).

The interaction between cells and collagen is mediated by membrane-anchored collagen receptors such as integrins, glycoprotein VI, leukocyte associated IG-like receptor-1, receptors from the mannose receptor family and Discodin Domain Receptors. Evidence is emerging that the functionality of collagen receptors and their affinity toward collagen are dependent on the fibrillar state of collagen. Four integrin heterodimers are known
to bind to collagen (α1β1, α2β1, α10β1, α11β1). Collagen affinity for both α1 and α2 domains is reduced by an order of magnitude when interacting with fibrillar collagen vs. monomeric collagen (Jokinen, 2004). The functionality of α1β1 integrin was lost in the presence of fibrillar collagen while the α2β1 integrin maintained its ability to mediate cell spreading and generate collagen gel contractions.

Glycoprotein VI (GPVI) is a collagen receptor present on platelets where it plays a central role in the formation of a hemostatic plug at sites of vascular injury (Ruggeri 2002). The adhesion of leukocytes to immobilized platelets is thought to contribute to inflammatory and thrombotic responses in damaged tissue. Studies of leukocytes captured by collagen-bound platelets showed that only fibrillar and not monomeric collagen type I was able to induce platelets activation via GPVI receptor (Butler, 2007). Platelets bound on monomeric collagen surfaces, are not activated and fail to capture leukocytes.

Three members of the mannose receptor family, mannose receptor (MR), the M-type phospholipase A2 receptor (PLA2R) and Endo180 (also known as uPARAP) are reported to mediate collagen endocytosis, which results in lysosomal degradation. Only mature collagen fibers and not monomeric collagen are reported to be incorporated into cytosolic vesicles following endocytosis (Everts, 1996). This suggests that fibrillar collagen has a higher affinity toward mannose receptors, as compared with monomeric collagen.
**Hypothesis**

Our hypothesis is that collagen binding to the DDR receptors is mediated by the fibrillar state of collagen, thus the DDRs collagen-binding affinity is different for monomeric and fibrillar collagen. To test this we propose to obtain a quantitative characterization of DDRs binding to collagen type I, by determination of the kinetic association and dissociation rates. Toward this purpose we will use Surface Plasmon Resonance (SPR) measurements that allow to study protein-protein interactions in real time, and to obtain a kinetic characterization of the process. Experiments will be performed using purified ECD of DDRs, which is necessary and sufficient for collagen binding. Even though not completely understood, DDRs oligomerization is reported to be important for collagen binding. Our DDR-ECD proteins are fused to the Fc region of human IgG1, which allows oligomerization via anti-Fc antibody. As a control for collagen binding specificity we will use an Fc fusion protein of the ECD of TrkB, the neurotrophin receptor for the brain-derived growth factor that does not bind to collagen.
2.2. MATERIALS AND METHODS

2.2.1. Materials

Our *in vitro* experiments utilized DDR1-and DDR2-Fc fusion proteins from Regeneron Pharmaceuticals (Tarrytown, NY). The entire ECD of mouse DDRs was fused to the hinge, C\textsubscript{H}2 and C\textsubscript{H}3 regions of human IgG1 via a bridging sequence (Gly-Pro-Gly) as previously described (Shrivastava 1997) and overexpressed in COS cells (diagram below).

**Diagram 2.1.** The structure of DDR-Fc fusion protein. (left); the Fc tag containing the C\textsubscript{H}2 and C\textsubscript{H}3 regions of the human IgG1 is shown in detail (right) together with the linker sequence. Cysteine residues (Cys) are shown in red.
The secreted DDR2-Fc fusion proteins were purified thereafter. An Fc fusion protein of the ECD of TrkB, the neurotrophin receptor for the brain-derived growth factor (Klein 1991), was used in place of DDRs as a negative control. In assays establishing DDR-collagen binding, it has been previously demonstrated that TrkB-Fc does not bind to collagens (Shrivastava 1997, Agarwal 2002). DDR-Fc or TrkB-Fc exist as a dimeric protein by virtue of their Fc tag (Stein 1998) and can be further oligomerized by means of anti-Fc antibody, which is expected to bring two DDR-Fc dimers together (diagram below).

Diagram 2.2. DDR-Fc complexes. a) DDR-Fc forms dimers via disulfide bonds in the Fc tag region; b) further clustering can be obtained using an anti-Fc antibody.
In our studies, DDR (or TrkB) oligomers were formed by incubating equal volumes (1:1) and identical concentrations of DDR-Fc and anti-Fc antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) at 4°C for 12 hours. Purified, pepsin-digested bovine dermal collagen type I was purchased from Vitrogen - Cohesion Technologies Inc. (Palo Alto, CA). This collagen is 95-98% type I collagen with the remainder being type III collagen. It is primarily in the monomeric form and contains < 1% oligomers.

2.2.2. SDS PAGE and Western Blotting

To determine the molecular weight of the purified DDR-Fc and TrkB-Fc fusion proteins, SDS PAGE was performed using 4-12% NuPage Novex Bis-Tris Gels from Invitrogen (Carlsbad, California). The proteins were diluted (to 10 ng) in NuPage LDS sample buffer (Invitrogen) containing 141 mM Tris base, 2% LDS, 10% Glycerol, 0.51 mM EDTA, 0.22 mM SERVA Blue G250, and 0.175 mM Phenol Red. Following SDS PAGE, the proteins were transferred onto nitrocellulose membrane (Invitrogen) and blocked in TBS-Tween buffer (Tris 20 mM, NaCl .5 M, pH 7.4-7.6, 0.05% Tween) with 5% milk. The membranes were then incubated overnight in TBS-Tween, 1% milk in the presence of 0.5 μg/ml anti-Fc antibodies at 4°C. The next day, the membranes were incubated with anti-goat IgG horseradish peroxidase (Santa Cruz, CA) and detection was performed using enhanced chemiluminescence (Amersham Biosciences). For accurate determination of the molecular weight, protein samples were electrophoresed on similar gels with BenchMark Protein Ladder (Invitrogen).
and stained with Coomassie type stain, Safe Stain (Invitrogen). Protein samples were also electrophoresed under reducing conditions by adding NuPage Sample reducing agent (Invitrogen) to the samples before electrophoresis.

2.2.3. Size Exclusion Chromatography (SEC)

To determine the percentage of oligomers formed by incubating DDR2-Fc fusion proteins with anti-Fc antibodies, an AKTA Purifier 10 Amersham FPLC system was used (Piscataway, NJ) with a Superdex 200 10/300 GL column from Amersham (Piscataway, NJ). The column was calibrated prior to sample injection with a mixture of ferritin [440 kDa], aldolase [158 kDa], ovalbulin [43 kDa], and ribonuclease A [13.7 kDa] by monitoring the absorbance of the eluant at 280 nm. Subsequently, DDR2-Fc or anti-Fc antibodies alone were injected at a concentration of 160 μg/ml and at a sample volume of 250 μl at 4°C with a flow rate of 0.4 ml/min. DDR2 oligomers were prepared by incubating 125 μl each of 320 μg/ml DDR2-Fc with 320 μg/ml anti-Fc antibody in PBS buffer and the resulting 250 μl of the sample volume was used for injection. Experiments were repeated at least two times under identical conditions and the area under the peaks was analyzed for calculating the percentage of DDR2-Fc incorporated into oligomers. Since the molecular weight of DDR1-Fc and TrkB-Fc was similar to DDR2-Fc (Figure 2.1.) and we expect that the mechanism of their oligomerization via anti-Fc antibody would be similar to that of DDR2-Fc, characterization of DDR1 and TrkB oligomers by size exclusion chromatography was not performed.
2.2.4. Surface Plasmon Resonance

Surface plasmon resonance (SPR) is a label-free technology for studying biomolecular interactions in real time (Stein 1998). SPR can provide answers about the specificity and strength of binding and the kinetic rate constants of binding and dissociation (BiaTechnology Handbook, 1999). This non-invasive technology is based on measurement of light reflected (SPR signal) from the side of a sensor surface that is not in contact with the sample. The SPR signal is very sensitive to the mass of biomolecules that bind to the sensor surface. One of the interactants (ligand) is initially immobilized to the sensor surface. When the other interacting molecule (analyte) is passed over it, molecules from the sample bind to the ligand-coated surface. Then, the local concentration changes and the SPR response is measured in response units (RU).

A Biacore 3000 system (Biacore Inc, Upsala Sweden) was used for SPR experiments. Gold-coated CM5 sensor chips were used to study DDR-collagen interactions. These sensor chips have a gold surface coated with a dextran matrix and allow immobilization of biomolecules using a large array of available coupling-chemistry systems. Above pH 3.5, the dextran matrix becomes negatively charged, so it is important to select the pH of the buffer containing the molecule to be immobilized, such that the molecule is attracted to the dextran matrix. Hence the isoelectric point of the biomolecule and its surface charge play an important role in the immobilization procedure.

Monomeric or fibrillar collagen type I could be immobilized on
the sensor surface using amine-coupling chemistry. The coupling procedure introduces N-hydroxysuccinimide esters into the surface matrix by modification of the carboxymethyl groups with a mixture of N-hydroxysuccinimide (NHS) and N-ethyl-N' (dimethyl-aminopropyl)-carbodiimide (EDC). These esters then react spontaneously with amines and other nucleophytic groups on the ligand to form covalent links. The coupling reaction requires uncharged amino groups on the ligand, so it is favored by high pH. After the injection of the ligand, ethanolamine is passed over the sensor surface to deactivate remaining active esters.

Monomeric collagen type I has an isoelectric point of over 8, therefore immobilization could be achieved using a buffer in pH range 6 to 7. For immobilization, monomeric collagen (10 µg/ml) in phosphate buffer (pH 6.0) was run at a flow rate of 5 µl/min for 1 min over the sensor surface. Fibrillar collagen (20 µg/ml), obtained by incubating monomeric collagen for 48 hrs at 37°C, was immobilized on a separate sensor surface using phosphate buffer (pH 7.0) at a flow rate of 5 µl/min for 6 min. The monomeric and fibrillar collagen surfaces generated using these protocols exhibited a surface of approximately 500 and 3000 RU, respectively. DDR1-Fc or DDR2-Fc monomers or oligomers were diluted in running buffer (HBS–EP from Biacore Inc) at concentrations ranging from 5 to 45 nM (0.65 to 12.5 µg/ml) and injected over the collagen surfaces at a flow rate of 2 µl/min for 10 min (association phase). A dissociation period of 15 min was allowed after each injection. After each analyte injection, the collagen surface was completely regenerated using two short pulses of 10 mM NaOH. A blank surface was created and DDR-Fc solutions were injected over it under
identical conditions to test for non-specific binding. As a negative control, TrkB-Fc monomers or oligomers were passed over the collagen surfaces under identical conditions.

SPR response curves obtained for DDR-Fc oligomer injections were used to determine their equilibrium dissociation constant ($K_D$) under a 1:1 Langmuir binding model. In this interaction model the association and dissociation phases are described by the following equations:

**Association phase:**

$$\frac{d[AB]}{dt} = k_a[A][B] - k_d[AB]$$

**Dissociation phase:**

$$\frac{d[AB]}{dt} = -k_d[AB]$$

**Equilibrium Dissociation Constant:**

$$K_D = \frac{k_d}{k_a}$$

Where, $[AB]$ is the concentration of DDR-collagen complexes, $[A]$ is the DDR-Fc oligomers concentration, $[B]$ is the collagen concentration, $k_a$ and $k_d$ are the association and dissociation kinetic rates, respectively.

The data obtained was fitted using BiaEvaluation 3.1 software. Due to the small dilution ratio of the analyte, an abrupt variation in the SPR signal occurred at the start and end of the injection, arising from the differences in running and analyte buffer. Those portions of the data were excluded in
the fitting procedure, as they did not contain any relevant information about the interaction studied. Also for DDR2-Fc injection, a small rise in signal could be seen at the end of the association phase. This occurred repeatedly during our experiments and we postulate that it is related to matrix effects, consisting in non-specific interaction with the dextran matrix itself (Bia Technology Handbook). Local fitting was used to improve the data fit, and the mean $K_D$ value was ascertained using a series of sample dilutions. For the flow rate used, the plot of the $\ln(d(\text{RU})/dt))$ was a straight line with a non-positive slope during the association phase (data not shown), characteristic of kinetic-limited data and indicated that mass-transfer limitation effects were not present. The residual plots were in the ± 2 RU range of noise level.

2.3. RESULTS

2.3.1. Characterization of DDR-Fc oligomers

To characterize the oligomeric state of the purified DDR-Fc proteins, we have used electrophoretic separation under reducing and non-reducing conditions. We have used unstained molecular weight markers and visualized the SDS PAGE gels with Coomassie staining for accurate determination of the molecular weight of the DDRs-Fc complexes. Under non-reducing conditions, DDR1-Fc was estimated to be ~ 197 kDa, DDR2-Fc ~ 190 kDa and TrkB- Fc ~ 220 kDa (Figure 2.1.). Under reducing conditions, DDR1-Fc was estimated to be ~ 91 kDa, DDR2-Fc ~ 90 kDa and TrkB ~ 110 kDa. Thus, our observations show that under non-reducing conditions the Fc fusion
proteins used in this study exist as dimers in solution. This is supported by the structure of the DDR-Fc protein (see Material and Methods, section 2.3.1.).

Figure 2.1. Electrophoretic characterization of Fc-fusion proteins. DDR1-Fc, DDR2-Fc and TrkB-Fc proteins were subjected to SDS PAGE and Coomassie staining. Molecular weight of DDRs-Fc and TrkB-Fc estimated under non-reducing (a, c) and reducing (b, d) conditions. As controls, we used samples containing only sample buffer and no without proteins.
To induce further oligomerization of the DDR-Fc fusion proteins we have used incubation with anti-Fc antibody. We used size exclusion chromatography (SEC) to determine the percentage of DDR-Fc oligomers obtained by incubating DDR-Fc proteins with anti-Fc antibodies under our experimental conditions. The anti-Fc antibody, DDR2-Fc alone (dimer), and the DDR2-Fc plus antibody solution (oligomer) gave elution volumes of 11.83, 10.59, and 8.08 ml, respectively (Figure 2.2.).

Figure 2.2. Size exclusion chromatography characterization of DDR2-Fc oligomers. Chromatograms of anti-Fc antibodies, DDR2-Fc dimers and DDR2-Fc oligomers injections are depicted above. By comparing the areas under the various curves peak we determined that 63% of DDR2-Fc dimers and 78% of anti-Fc antibodies are incorporated into DDR2-Fc oligomers.
Examination of the oligomer injection chromatogram indicates that under our experimental conditions there is a residual amount of pure DDR2-Fc and anti-Fc antibody. When comparing the SEC results from DDR2-Fc pure protein (dimer), anti–Fc antibody alone and DDR2-Fc oligomer injections, we observed an approximately $63 \pm 4\%$ shift from the dimer state to the oligomer state. This value was obtained by comparing the area under the dimer peak in the DDR2-Fc injection with the area under the dimer peak in the DDR2-Fc oligomer injection wherein both injections contained the same amount of DDR2-Fc protein (40 μg). When comparing the area under the antibody peak in the pure antibody injection to the area under the antibody peak in the DDR2 oligomer we observed a $78 \pm 4\%$ transfer of the antibody to the oligomer formation.

As the DDR-Fc ECD does not have a globular shape, the elution volume cannot be used to determine the molecular mass. Nevertheless, considering the percentages of the DDR-Fc dimer population and anti-Fc antibody population incorporated into the DDR-Fc oligomer complexes, we propose that each DDR-Fc oligomer is likely comprised of two DDR-Fc (dimeric) proteins bound to one anti-Fc antibody with one or two additional antibody molecules attached to an unbound Fc region of a DDR-Fc dimer (Diagram 2.3). This oligomeric stoichiometry and molecular weight of DDR-Fc dimers (determined using Western blotting) have been used to calculate the molarity of DDRs in oligomeric solutions, and to determine their dissociation constants by SPR.
Diagram 2.3. Stoichiometry of DDR-Fc oligomers. Incorporation of 63% of the DDR-Fc dimers and of 78% of the anti-Fc antibodies into the DDR-Fc oligomer complexes will result in a mixture of two species with the DDR-Fc and anti-Fc antibody stoichiometry depicted above.

2.3.2. **DDR-Fc requires oligomerization for binding to collagen type I**

To investigate DDR-Fc binding to collagen type I, SPR studies were performed, in which DDR1-Fc (or DDR2-Fc) dimer or oligomer solution was passed over the same collagen surface in successive injections after regeneration. As a control, TrkB-Fc dimers and oligomers prepared using identical protocols were passed over the same collagen surface, and no significant binding response was observed (Figure 2.3.). Figure 2.4., shows that for the same collagen surface the binding response (RU) increases fourteen times for DDR1-Fc oligomers compared to DDR1-Fc dimers; and eight times for the DDR2-Fc oligomers compared to the DDR2-Fc dimers. The response of
DDR-Fc dimers was too small to determine the equilibrium dissociation ($K_D$) constants in these experiments. During the injections of dimeric DDR-Fc, a slight increase of the sensor response after the analyte injection is observed, in contrast with the usual decrease in the signal during the dissociation phase. This can be explained by reorientation of the dextran matrix during the analyte injection.

**Figure 2.3.** SPR binding controls. SPR experiments demonstrating a ‘flat’ response for monomeric and oligomeric TrkB-Fc injected over a collagen surface; DDR2-Fc oligomers exhibit positive binding response when injected over the same collagen surface.
Figure 2.4. DDRs-Fc binding to collagen type I. Surface plasmon resonance (SPR) experiment following the binding of DDR1-Fc and DDR2-Fc to immobilized collagen type I. DDR1-Fc (upper graph) and DDR2-Fc (lower graph) dimers or oligomers (22 nM) were injected over the same collagen surface. DDR-Fc dimers alone failed to show any binding response while DDR-Fc oligomers showed a large binding response.
2.3.3. Collagen fibrillar state modulates DDRs binding affinity

SPR experiments were performed to determine the kinetic parameters of DDR-Fc oligomers binding to collagen type I, in monomeric and fibrillar state (Table 2.1. and Figure 2.5.). SPR is especially suited for studying the kinetics of protein-protein interactions due to the sensitivity of the detector and the possibility to follow the process in real time. Monomeric and fibrillar collagen type I surfaces were created, and DDR-Fc oligomers were injected over them in the molarities indicated. The molarity of the DDR-Fc oligomers was calculated by considering one DDR-Fc oligomer (consisting of four DDR-Fc monomers) as one unit. Data were fitted using a 1:1 Langmuir binding model (as detailed in the Materials and Methods section 2.3.4.) to determine the $K_D$ constant – that is inversely proportional with the binding affinity.

<table>
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<th>Fibrillar collagen $K_D$ (nM)</th>
<th>Monomeric collagen $K_D$ (nM)</th>
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Table 2.1. Kinetic constants of DDR-collagen binding.
Figure 2.5. Collagen fibrillar state mediates DDRs binding. Surface plasmon resonance data of oligomeric DDR1-Fc (a, c) and DDR2-Fc (b, d) binding to immobilized monomeric (upper row) and fibrillar (lower row) collagen type I. DDR1-Fc and DDR2-Fc oligomer solutions were injected at concentrations of 45, 33, 24, 14, 7 and 45, 31, 23, 14, 7 nM respectively, top to bottom. The calculated K_d values are indicated in the figure. The binding levels for various DDR-Fc concentrations are described in Table 2.2.
DDR1-Fc and DDR2-Fc have distinct binding characteristics for monomeric vs. fibrillar collagen. Both DDR1-Fc and DDR2-Fc oligomers showed an increased affinity for monomeric vs. fibrillar collagen. One can also note the significant difference in the DDR’s binding response to collagen in the monomeric state vs. the fibrillar state (Table 2.2.). To obtain the normalized response for fibrillar surface in Table 2.2., we used the concept that the binding of the analyte to the ligand surface is directly proportional with the size of the ligand surface. Accordingly, the normalized response for the fibrillar-collagen surface was obtained by dividing the original values to the ratio between the fibrillar and monomeric surfaces.

<table>
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<th>Fibrillar-collagen surface response (RU)</th>
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<th>Fibrillar-collagen surface response (RU)</th>
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<tr>
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</tr>
<tr>
<td>7</td>
<td>-</td>
<td>73</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 2.2. DDR1-Fc and DDR2-Fc binding to monomeric and fibrillar collagen.
2.4. DISCUSSION

Characterization of purified DDR-Fc proteins showed that they exist in solution predominantly as dimers. This is in agreement with results reported by others using Fc fusion proteins (Klein 1991, Shrivastava 1997) that the Fc tag induces dimerization via disulfide bonds between cysteine residues in the Fc region. SEC experiments performed to characterize the oligomerization of purified DDR ECD via anti-Fc antibody, showed that 63% of DDR2-Fc and 78% of anti-Fc antibody is incorporated in the oligomer complex. Based on these results we propose that each DDR-Fc oligomer is likely comprised of two DDR-Fc (dimeric) proteins bound to one anti-Fc antibody with either one or two additional antibody molecules attached to an unbound Fc region of a DDR-Fc dimer. This oligomeric stoichiometry and molecular weight of DDR-Fc dimers (as determined by Western blotting) have been used to calculate the molarity of DDRs in oligomeric solutions and to determine their dissociation constants by SPR.

It has been reported that the ECD of DDRs requires dimerization and/or oligomerization for binding to collagen. Here we have established using SPR that antibody-mediated oligomerization of both DDR1-Fc and DDR2-Fc significantly enhances their binding to immobilized collagen type I. Our results are consistent with earlier observations using the identical fusion proteins; namely, that SPR was unable to detect any binding response of DDR2-Fc proteins alone with collagen (Shrivastava 1997) and microplate-based assays showed results consistent with our SPR data (Agarwal 2002). Utilizing a
different fusion construct (DDR2-His), Leitinger has reported that dimerization of DDR2 is necessary and sufficient for its binding to collagen (Leitinger 2003). These observations are in slight contradiction with our results as Leitinger reports that His-DDR2 (which is a dimer) is able to bind to collagen with no further oligomerization. It is possible that the difference between the dimer structure formed by the DDR-Fc and DDR-His accounts for the observed variation in collagen binding. As depicted in the diagram bellow, the relative orientation of the individual Discoidin Domains (DS) in the DDR-Fc and DDR-His can be different when the proteins exist in a dimeric state; anti-Fc antibody oligomerization of DDR-Fc proteins may be required to bring two of the individual DS components of the oligomer into the proper orientation for high affinity binding to collagen.

**Diagram 2.4.** Structural effects on collagen binding. Relative orientation of individual DS domains is described by the angle $\theta$ and $\alpha$ in the case of DDR-Fc dimer and by the angle $\theta'$ and $\alpha'$ in the case of DDR-His dimer. Oligomerization of DDR-Fc via anti-Fc antibodies may rearrange the DS domains into $\theta'$ and $\alpha'$ orientation.
The equilibrium dissociation constants ($K_D$) of DDR1-Fc and DDR2-Fc oligomers were nearly identical suggesting an equal affinity for collagen. At the same time we observed an increase in the total binding response for DDR1-Fc as compared to DDR2-Fc, for the same collagen surface, suggesting that the collagen-binding mechanism may be different for the two receptors. This is in agreement with previous observations by others (Abdullhussein 2004), where DDR1 required the entire extracellular domain for binding to collagen type I, while only the DS region was sufficient for DDR2 binding to collagen type I. Even though the homology in the DS region for DDR1 and DDR2 is $\sim 60\%$, the stalk region has only $44\%$ homology. Mutational analysis experiments also revealed a difference in the set of loops critical for collagen binding: L1, L3 and L4 in the case of DDR1 and L1, L2 and L4 in the case of DDR2. Therefore, it is not surprising that the collagen-binding mechanism may be distinct between DDR1 and DDR2.

Our SPR results indicate that DDRs can clearly distinguish between the monomeric and fibrillar states of collagen with an increased affinity toward monomeric collagen. In our SPR experiments when immobilizing collagen fibrils, some amount of collagen monomers also becomes immobilized on the sensor surface, thus the actual affinity of DDRs to fibrillar collagen may be even lower. For both DDR1-Fc and DDR2-Fc oligomers the affinity toward fibrillar collagen was an order of magnitude lower as compared with the affinity toward monomeric collagen. These results may suggest that the structure of the DDR binding site
on collagen is altered by the incorporation of the collagen monomers into fibers.

It is interesting to note that the same decrease in binding affinity is observed for integrins when transitioning from the monomeric toward the fibrillar state of collagen. In the case of integrins, this effect was speculated to play a role in pericellular collagen fiber formation, by creating an increase in the local concentration of collagen monomers (Jokinen 2004). As the monomers are incorporated into fibers, the affinity toward bound integrins decreases and the fibers are released. This model can also be potentially applied to the DDR-collagen interaction.

At the same time the total binding response was lower for the fibrillar-collagen surface vs. the monomeric-collagen surface. This observation indicates that collagen incorporation into fibers induces a decrease in the number of sites available for DDR binding. This interpretation is supported by the radial growth of collagen fiber – i.e. the collagen monomers at the fiber core are not available for DDR binding.

In conclusion, determination of the kinetic constants characterizing the DDR-collagen interaction, confirmed our hypothesis that the fibrillar state of collagen modulates its affinity toward DDR receptors. Taken together, these results suggest that DDRs can function as a cellular probe for the state of the extracellular matrix and provides new insights in the ECM-cell interaction.
CHAPTER 3

THE OLIGOMERIC STATE OF DDR1 ON THE PLASMA MEMBRANE MEDIATES ITS INTERACTION WITH COLLAGEN

3.1. INTRODUCTION

Background

The role of DDR1 oligomerization in ligand binding and subsequent phosphorylation, though considered important, is only partly understood. *In vitro* work by our laboratory (Agarwal 2007) and by others (Leitinger 2003) revealed that high affinity interaction with collagen requires dimerization and/or oligomerization of DDR1. Two independent reports (Noordeen 2006, Abdulhussein 2007), showed that a significant percentage of the DDR1 population forms ligand-independent dimers, and this percentage is not changed upon collagen stimulation.

While in the commonly accepted mechanism of Receptor Tyrosine Kinases (RTKs) activation, ligand binding induces a transition from monomeric to dimeric/oligomeric state, recent reports have come to challenge this as a general assumption. Specifically Epidermal Growth Factor Receptor (EGFR) was found to form ligand-independent dimers (Moriki 2001, Martin-Fernandez 2002,
In the case of the EGFR receptor, it is reported that two distinct affinity classes are present, and while not confirmed, it is thought that they are associated with different oligomerization states of the receptor (Wiedlocha 2004). Similarly, the insulin receptor forms disulfide-linked dimers that are activated without further oligomerization (Ottensmayer 2000). In cytokine receptors, which are believed to share many mechanistic features with RTKs, the ligand–induced dimerization model has recently been challenged by the findings that both erythropoietin and the growth hormone receptors exhibit ligand-independent dimerization (Gent 2002, Brown 2005).

It is well understood that following ligand binding many RTKs undergo redistribution in, or around, the plasma membrane. Lipid raft microdomains are membrane domains that compartmentalize the cell membrane. They consist of a dynamic assembly of cholesterol and glycosphingolipids that form ordered liquid domains floating in the less ordered liquid domains of the surrounding membrane (Simons, 2000). It is thought that lipid rafts have the ability to recruit, as well as exclude, specific lipids and proteins and have been implicated in the regulation of various physiological processes such as lipid sorting, protein trafficking, cell polarization and signal transduction (Hancock 2003, Parton 2004). EGFRs have been found to localize in lipid rafts prior to ligand binding. After ligand binding, the levels of EGFR in the rafts were found to decrease, and this decrease was demonstrated to depend on RTK activity (Mineo 1999). Also, for the EGFR receptors, ligand stimulation was reported to induce internalization and incorporation into cytosolic vesicles (LeRoy 2005).
Focal adhesions are large, dynamic protein complexes, through which cells connect to the ECM. They serve as mechanical linkages to the ECM, as well as a biochemical signaling hub to concentrate and direct numerous signaling proteins. Collagen-binding integrins, are redistributed and recruited to focal adhesion (FA) sites as the cell undergoes cytoskeletal reorganization during cell migration (Hynes 2002).

**Hypothesis**

DDR oligomerization was shown to be necessary for *in vitro* binding to collagen. Our hypothesis is that the oligomeric state of DDR on the plasma membrane mediates its interaction with collagen. To test this we propose to use Fluorescent Resonant Energy Transfer (FRET) microscopy that will allow mapping of the DDR oligomeric state on the plasma membrane, and thus allow us to differentiate between the monomeric and oligomeric fractions of the DDR population. For the FRET experiments we will use recombinant DDR1 tagged with variants of the Green Fluorescent Protein (GFP), Yellow Fluorescent Protein (YFP) and Cyan Fluorescent Protein (CFP). Receptors from the RTK family are reported to undergo redistribution in/around the plasma membrane following ligand binding. We propose that collagen binding modulates the distribution of DDR receptors. To test this hypothesis we will use live cell microscopy that will allow us to follow the effects of collagen stimulation on the fluorescently labeled DDR in real time.
3.2. MATERIALS AND METHODS

3.2.1. Reagents

Anti-DDR1 antibody sc-532, anti-GAPDH antibody, anti-mouse and anti-rabbit IgG, HRP conjugated antibodies were purchased from Santa Cruz Biotech, (Santa Cruz, CA). Protein A coated agarose beads were purchased from Invitrogen (Carlsbad, CA). Bovine dermal collagen type I was purchased from Invitrogen. Glass bottom culture dishes for live cell microscopy were purchased from MatTek Glassware (Ashland, MA). *NheI*, *Eco47III* and *EcoRI* restriction enzymes were purchased from New England Biolabs (Ipswich, MA). *PfuTurbo* polymerase and CIAP were purchased from Stratagene (LaJolla, CA).

3.2.2. DNA constructs

A plasmid containing the entire mouse DDR1b sequence was obtained from Regeneron Pharmaceuticals (Tarrytown, NY). To generate the DDR1-CFP construct, we have used the Cerulean variant of the pECFP-C1 vector, which was a generous gift of Dr. Dave Piston (Vanderbilt University, Nashville TN) (Rizzo 2004). This plasmid was linearized by restriction digestion with *Eco47III* and phosphatased using standard procedures. The DDR1 coding region was amplified by polymerase chain reaction (PCR) utilizing the following primers: 5’ primer was 5’–Phos-ACCATGGGGACAGGGACCCTC-3’ and the 3’ primer was 5’-Phos-CACCGTGGTGGAGGCGCATCATCC-3’; the resulting PCR product was blunt-end ligated immediately upstream of the CFP open reading frame. To generate the DDR1-YFP construct, we used the pEYFP-N1
vector from Clontech (Carlsbad, CA). The DDR1 coding region was amplified by PCR using primers that introduced a *Nhe*I restriction site on the 5' end: 5'-AGAGG GTTCCGCTAGCGCCACCATGGGGACAGGGACCCTCTCATC-3' and an *Eco*RI restriction site at the 3' end: 5'-GTCGACTGCAAGATTGCACCGGTGGAGCG CATCATCCG-3'. The PCR products were double digested with *Nhe*I and *Eco*RI and directionally ligated into the *Nhe*I and *Eco*RI restriction sites of the pEYFP-N1 vector. The authenticity (i.e. correct orientation and in frame with either the CFP or YFP coding region) of the resulting clones was verified by Sanger dideoxynucleotide sequencing. A pEGFP-C3 vector for expression of N-terminus GFP labeled Rab5a was a kind gift from Dr. Heidi McBride (University of Ottawa Heart Institute, Canada).

### 3.2.3. Cell samples preparation

**Substrates used for cell-culture**

Three different cell types (HEK 293, COS 7, and 3T3) were used based on their suitability for FRET or confocal microscopy or SDS PAGE analysis. For SDS PAGE experiments, HEK 293 cells were seeded on NunClon culture dishes; for widefield or FRET microscopy experiments, 3T3 cells were seeded on 20 mm glass bottom culture dishes and for confocal microscopy COS 7 cells were seeded on 25 mm glass cover slips. The factors determining the cell-type selection were based on cell adherence to the substrate, level of protein expression after transient transfection and cell thickness.
**DDR1 activation experiments**

HEK 293 cells from ATCC (Manassas, VA) were grown in Dulbecco's Modified Eagle Medium (DMEM) from Gibco, supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (pen-G 10,000 units/ml; streptomycin 10,000 µg/ml; amphotericin B 25 µg/ml). This cell line does express DDR1, however in the biochemical assays we were able to distinguish between the endogenous DRR1 and DDR1-YFP fusion proteins, as the latter has a higher molecular weight. In the microscopy experiments, only the DDR1 fusion proteins were analyzed, as the endogenous DDR1 is not detectable through fluorescence microscopy imaging. The cells were transiently transfected with DDR1-CFP or DDR1-YFP using Fugene transfection reagent (Roche, Basel Switzerland). After 24 hours of transfection the cells were serum starved overnight and stimulated with 10 µg/ml collagen type I for time intervals ranging from 5 min to 2 hrs. Following stimulation, the cells were lysed and subjected to SDS PAGE. Alternatively, the cells were washed with PBS (supplemented with Ca$^{2+}$ and Mg$^{2+}$ ions) and imaged live. Nonstimulated control samples were also prepared.

**FRET microscopy experiments**

Mouse osteoblast 3T3 cells obtained from ATCC were cultured in Minimal Essential Medium Alpha (MEM alpha) from Gibco, supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (pen-G 10,000 units/ml; streptomycin 10,000 µg/ml; amphotericin B 25 µg/ml). Samples
were transfected with DDR1-YFP and/or DDR1-CFP and stimulated with collagen as described above. The cells were then washed with PBS and imaged live using FRET microscopy. These cells do express low levels of endogenous DDR1 which is not detectable with fluorescence microscopy and did not interfere with the FRET experiments.

Confocal microscopy experiments

African Green monkey fibroblast COS 7 cells from ATCC were cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic (pen-G 10,000 units/ml; streptomycin 10,000 µg/ml). Cells seeded on glass coverslips were transiently transfected with DDR1-CFP, Rab5a-GFP or both and stimulated with collagen as described above. Following stimulation, the cells were washed three times with PBS and fixed with 2% paraformaldehyde for 30 min. Following fixation the coverslips were mounted on microscope glass slides using Prolong mounting media from Invitrogen. Similar samples were prepared using HEK 293 cells, transfected with DDR1-YFP wherein, following fixation, the cells were also nuclear stained with 4 µM bisbenzimide from Calbiochem (San Diego, CA).

3.2.4. SDS PAGE and Western Blotting

Following stimulation, HEK 293 cells were lysed in 20 mM Tris-HCl (ph 7.8), 150 mM NaCl, 2 mM sodium orthovanadate, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin 10 µg/ml leupeptin and 2 mM...
EDTA. Whole cell lysate was analyzed using SDS PAGE performed as previously described (Mihai 2006). Briefly, samples containing 15 μg of protein sample were prepared in 1x NuPage LDS sample buffer (Invitrogen, Carlsbad, California). Electrophoresis was performed using 4–12% (w/v) NuPAGE Novex Bis-Tris Gels from Invitrogen. BenchMark Protein Ladder (Invitrogen) was used as marker. SDS PAGE was followed by Western blotting using nitrocellulose membranes (Invitrogen). The membranes were probed with anti-DDR1 antibodies and imaged using enhanced chemiluminescence from Amersham Biosciences (Piscataway, NJ) after incubation with HRP-conjugated anti-rabbit IgG.

Alternatively, cells were lysed in 20 mM Tris-HCl (ph 7.5), 150 mM NaCl, 2 mM sodium orthovanadate, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin 10 μg/ml leupeptin and 2 mM EDTA. DDR1 was immunoprecipitated (IP) from the whole cell lysate, using anti-DDR1 antibodies and protein A coupled agarose beads. Following overnight incubation, the IP reactions were washed three times in lysis buffer and the protein separated from the beads by boiling for 5 min in 1x sample buffer. The resulting samples were analyzed for phosphorylation using SDS PAGE, followed by Western blotting and incubation with anti-phosphotyrosine antibodies (Cell Signaling Technologies, Boston MA).
3.2.5. Microscopy

Colocalization studies between Rab5a–GFP and DDR1-CFP were performed using a Zeiss LSM 510 confocal microscope. GFP was imaged using the 488 line of the Argon laser, a HFT KP 700/488 beam splitter and a 500–550 IR BP filter. The CFP was imaged using the 458 line of the Argon laser, a HFT 458/514 beam splitter and a 480/520 BP filter. Images were acquired using a 63X objective, with an acquisition rate of 12 kHz and 1024 x 1024 pixel resolution. To minimize the noise an average of 8 scans per line was used. To establish eventual bleedthrough between the GFP and CFP channels, we imaged control samples containing cells expressing only DDR1-CFP or only Rab5a-GFP. Under these imaging conditions, no bleedthrough was detected between the CFP and GFP channels and elimination of out-of-focus fluorescence allowed for clear colocalization analysis. Imaging of fixed HEK 293 cell samples was performed using the 514 line of the Argon laser, a HFT 458/514 beam splitter and a 530 IR long pass filter. The nuclear stain was imaged with a tunable Ti-Sapphire laser at 750 nm in two photon mode.

FRET experiments and internalization dynamics studies were performed in wide field microscopy, using a Zeiss Axiovert 200 equipped with an Excite 120 light source from Excite Mississauga, Canada and a cooled charged-coupled device (CCD) ORCA camera from Hamamatsu, Bridgewater, NJ. The FRET experiments were performed in an acceptor sensitizing set up (Gordon 1998, Zal 2004), directly measuring the emission of the acceptor while exciting the donor. Imaging was performed in a three filter cube set–up, using a 63X
water immersion objective. The optical filters are from Semrock, (Rochester, NY), I_{DD} – channel, donor excitation – donor emission (excitation 438/24, emitter 475/25), I_{AA} – channel, acceptor excitation – acceptor emission (excitation 500/24, emitter 542/27) and I_{DA} – channel, donor excitation – donor emission (excitation 438/24, emitter 542/27). The internalization dynamics studies were performed using the YFP labeled receptor, with imaging performed in the acceptor channel described above.

### 3.2.6. FRET analysis

We used the nomenclature proposed by Zal and Gascoine, 2004. The following bleedthrough constants were measured on samples containing the acceptor only (A) or donor only (D) expressing cells – indicated in the brackets near the channel index:

\[
\begin{align*}
    a &= \frac{I_{DA}(A)}{I_{AA}(A)} \\
    b &= \frac{I_{DD}(A)}{I_{AA}(A)} \\
    c &= \frac{I_{AA}(D)}{I_{DD}(D)} \\
    d &= \frac{I_{DA}(D)}{I_{DD}(D)}
\end{align*}
\]  

(eq. 1)

FRET imaging was performed on samples expressing the donor and acceptor, and the corrected sensitized fluorescence, \( F_C \) was calculated according to the formula below:

\[
F_C = I_{DA} - a \cdot (I_{AA} - c \cdot I_{DD}) - d \cdot (I_{DD} - b \cdot I_{AA})
\]  

(eq. 2)
After image acquisition, bleedthrough parameters were derived using PixretFret, an ImageJ plugin, proposed by Feige et al., (2005). This allowed for pixel-by-pixel analysis, and determination of the above constants for a large range of intensity values. The minor bleedthrough constants, $c$ and $b$, were found to be negligible in our set-up and henceforth were ignored in our calculations. We found $a = 0.150$ and $d = 0.400$; those values were constant across the entire range of intensities found in our samples, except for the very low range, that exhibited large fluctuations in the constants values. Those regions were excluded from our analysis. Equation 2 thus becomes:

\[ F_C = I_{DA} - a \cdot I_{A4} - d \cdot I_{DD} \]  

(eq. 3)

The corrected FRET signal was normalized against the acceptor fluorescence, the resulting FRET index being shown to linearly depend on both the transfer efficiency and the number of acceptor – donor pairs (Zal 2004):

\[
\frac{F_C}{A} = \frac{\varepsilon_{D1}}{\varepsilon_{A1}} a E_{FRET} \frac{N_{ad}}{A} , \text{ or } \frac{F_C}{A} = \alpha E_{FRET} \frac{N_{ad}}{A} \]  

(eq. 4)

Where, $A$ is the number of acceptor labeled receptors; $\varepsilon_{D1}$, $\varepsilon_{A1}$ are the absorbance coefficients at donor excitation wavelength of donor or acceptor, respectively; $a$ is the bleedthrough constant defined above; $N_{ad}$ is the number of acceptor donor pairs, and $E_{FRET}$ is the FRET transfer efficiency for the current structure of the donor and acceptor labeled DDR1 dimer; $\varepsilon_{D1}$, $\varepsilon_{A1}$ and $a$ are
constant for our experimental set up, and can be written as: \( \alpha = \frac{\varepsilon_{D1}}{\varepsilon_{A1}} \). In our numerical analysis, \( A \) is given by the signal intensity in the \( I_{AA} \) channel and \( D \) is given by the signal intensity in the \( I_{DD} \) channel.

We considered that the percentage \( x \), of the receptor population that exists in dimeric state can vary from the stimulated to the nonstimulated samples. Since the acceptor- and donor-labeled receptors are identical with respect to their cellular location and function, we considered the probability of forming acceptor-donor labeled dimers (\( \text{P}_{ad} \)) to be equal with the probability of forming acceptor-acceptor (\( \text{P}_{aa} \)) and donor-donor labeled dimers (\( \text{P}_{dd} \)). So, the number of acceptor-donor labeled pairs is given by:

\[
N_{ad} = x \cdot D \cdot \text{P}_{ad} = x \cdot D \cdot \frac{x \cdot A}{x \cdot (A + D)} = x \cdot \frac{A \cdot D}{A + D} 
\]

(eq. 5)

Considering the ratio between acceptor and donor labeled receptors \( r = A / D \),

\[
N_{ad} = x \cdot \frac{A}{1 + r} 
\]

(eq. 6)

Combining relations 4 and 6, the FRET index can be written as:

\[
\frac{F_{C}}{A} = \alpha (E_{FRET} \cdot x) \cdot \frac{1}{1 + r} 
\]

(eq. 7)
3.2.7. Image analysis

Quantitative evaluation of the microscopy data was performed in ImageJ. For the FRET experiments measurements were performed on defined cellular region of interest (ROIs). The mean fluorescent intensity was derived with ImageJ and subsequent numerical calculations and statistical analysis was performed in Microsoft Excel according to the equations presented above.

To evaluate the percentage of internalized receptor in the internalization kinetics experiments, we defined two ranges of receptor intensities: 0 – 5,000 in arbitrary units (AU) for the noninternalized receptor, and 5000 – 17,000 AU for the internalized receptor. Those intervals were determined by analysis of both stimulated and nonstimulated samples. The integrated intensity encompassed by either of those ranges was measured and equated with the fraction of the receptor population being internalized or noninternalized.

3.3. RESULTS

3.3.1. DDR1-YFP receptors undergo collagen-induced phosphorylation

To study DDR1 distribution and oligomeric state in the plasma membrane, we have generated expression constructs that result in the synthesis of DDR1-YFP and DDR1-CFP fusion proteins (Figure 3.1.a). These DDR1-GFP proteins exhibited a MW higher than the MW (120KD) of native DDR1 (Figure 3.1.b).
Figure 3.1. DDR1-YFP receptors are functional. (a) Schematic representation of the DDR1-YFP fusion proteins. (b) HEK 293 cells, native or transiently transfected with DDR1-YFP, were stimulated with 10 µg/ml collagen type I for 90 min (as indicated) and the cell lysates immunoprecipitated with anti-DDR1 antibodies; following SDS-PAGE and Western blotting, the membranes were probed with anti-phosphotyrosine antibodies - anti-py (upper blot) or anti-DDR1 antibodies (lower blot). Collagen stimulation increases the phosphorylation level of the fluorescently labeled DDRs.
To verify that our DDR1 fusion proteins underwent collagen-induced receptor activation like the native DDR1, we detected the phosphorylation of DDR1-YFP(s) upon collagen stimulation using immunoprecipitation, SDS PAGE and Western blotting. As shown in Figure 3.1.b, the collagen stimulated DDR1-YFP (and CFP, data not shown) samples showed increased phosphorylation levels as compared with nonstimulated controls. The level of DDR1-YFP in each lane was checked by reprobing the blots with anti-DDR1 antibody

3.3.2. DDR1 exists as a dimer before collagen binding

We studied the oligomeric state of DDR1 using FRET experiments in live 3T3 cells. As described in the Methods section we used a sensitized acceptor set-up. First, cells were transiently transfected with either DDR1-YFP or DDR1-CFP alone, to determine the bleedthrough constants a, b, c, d according to eq. 1. Next, cells co-transfected with both DDR1-CFP and DDR1-YFP were imaged live using widefield fluorescence microscopy. The extensive cell spreading generated a uniform distribution of the fluorescent labeled DDR1 across the cell surface (Figure 3.2.). The level of expression for both constructs varied substantially from cell to cell even in the same sample. In our analysis we used a FRET index that is obtained by normalizing the corrected FRET signal, to the acceptor levels. The FRET index was found to be stable for small fluctuations in the acceptor/donor ratio (A/D) < 0.25, and independent of the acceptor levels.
Figure 3.2. FRET analysis of DDR1 dimerization. 3T3 cells transiently transfected with both DDR1-YFP and DDR1-CFP were imaged live. For the same region, images were acquired sequentially in the $I_{AA}$, $I_{DD}$ and $I_{DA}$ channels (a, b, c); the corrected and normalized FRET signal is displayed in pseudocolor (d). Positive FRET signal is indicative of dimer formation and is uniformly recorded over the entire cell surface. Perinuclear regions that display higher signal intensity were found to have abnormal acceptor to donor ratio. All scale bars are 20 μm.
Nonstimulated samples exhibited positive FRET values, indicating that the members of the DDR1 FRET pair come in close proximity ~ less than 100 Å (Figure 3.2.). As this result is not dependent on the level of protein present in the samples (Figure 3.3.a), we conclude that part of the DDR1 population exist as dimers prior to collagen binding. This is consistent with reports (Nordeen 2006, Abdullhusein 2007), that found DDR1 to form dimers independent of collagen. Mapping of the FRET index over the entire cell surface showed that the DDR1 dimers are homogeneously distributed.

To further verify the robustness of our FRET analysis we plotted the FRET index as a function of A/D (eq. 7) (Figure 3.3.b). It is reported (Pentcheva 2001), that the FRET index is dependent on A/D only for clustered acceptor distributions and independent of this ratio for random acceptor distributions. In our experimental set-up we had no direct control on the absolute and the relative expression of the A/D. When analyzing the samples across the entire range of A/D ratio, we observed a decrease in the donor levels with the increase in A/D ratio. This would result in limiting of the maximum number of acceptor-donor pairs regardless of the increase in A/D. To counteract this effect we normalized the slope of the FRET index vs. A/D graph to the slope of the donor levels vs. the A/D graph (Figure 3.3.b). Our results showed that indeed, the FRET index depends on A/D with a positive slope, confirming that the FRET signal is generated by a specific interaction between the different labeled receptors.
Figure 3.3. Analysis of FRET index distribution. (a) The FRET index was plotted against the acceptor levels for nonstimulated samples and for aggregated regions in the stimulated samples as indicated; in both sets of data the FRET index shows little dependence on the receptor levels. (b) FRET index plotted against $1/(1+r)$ as described in eq. 7, for nonstimulated and stimulated cells. The slope derived from the stimulated samples is higher than the one derived from the nonstimulated sample, consistent with the increase in the FRET index.
3.3.3. Collagen induces DDR1 aggregation and an increase in FRET signal

Collagen stimulation was found to induce aggregation of DDR1 into a clustered distribution (Figure 3.4.c). As can be seen in the Figure 3.4.d, there is a sharp increase in the FRET signal from the nonstimulated to the collagen stimulated samples that is confined to the regions of aggregated receptor. The regions from the stimulated samples, containing nonaggregated DDR1, showed a slight decrease in FRET. Nevertheless, the DDR1 concentration in those regions exhibits intensity values that fall in the noise range of FRET analysis, thus making it difficult to accurately evaluate the FRET index there. As a result we confined our quantitative evaluation of the FRET signal in collagen stimulated samples, on the regions of DDR1 aggregation. As expected, the average receptor density (reflected in the acceptor and donor intensity) was higher in the regions of aggregation as compared to the average density in the nonstimulated samples. We found the FRET index to be constant across a wide intensity range in the regions with aggregated DDR1. Moreover, the lower end of the intensity range in the regions of aggregation (stimulated samples) is overlapping with the upper end of the intensity range in regions of uniform distribution (nonstimulated samples), so we were able to compare the FRET index at the same receptor levels (Figure 3.3.a). The difference in the FRET index was found to be conserved, confirming that this result is not an artifact of higher receptor concentrations.
Figure 3.4. Collagen induces DDR1 aggregation followed by an increase in FRET signal. 3T3 cells co-transfected with DDR1-YFP and DDR1-CFP were imaged before and after stimulation with collagen type I (as indicated); upper row shows images in the YFP channel ($I_{AA}$), demonstrating uniform receptor distribution in the nonstimulated sample (a); collagen stimulation induces aggregation of DDR1 (c); lower row shows the FRET analysis of the images in the upper row. The stimulated sample (d) exhibits higher FRET values in the regions of aggregated DDR1. The ROI indicated by white squares in the upper row images were magnified in both sets of images for clear demonstration of receptor aggregation and increase in FRET; all scale bars are 20 µm.
Analysis of the FRET index vs. A/D graph for the stimulated samples, also showed a positive slope that was higher than the one derived from the nonstimulated sample (Figure 3.3.b). This is consistent with eq. 7, which shows the slope of the FRET index vs. A/D graph to be directly proportional with the product between the transfer efficiency and the dimer state occupancy. We have quantified the increase in FRET index for samples stimulated with collagen for various time intervals ranging from 5 to 30 min. The FRET values increased significantly from the nonstimulated samples to the stimulated samples, from 5.4 ± 1.3 to 15.0 ± 2.6, as early as 5 min after collagen stimulation (Table 3.1.). The measured FRET index did not show any significant difference between the various intervals of collagen stimulation thereafter.

<table>
<thead>
<tr>
<th>Collagen stimulation</th>
<th>nonstimulated</th>
<th>5 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F_C / A$</td>
<td>5.4 ± 1.3</td>
<td>15.0 ± 2.6</td>
<td>14.1 ± 2.7</td>
</tr>
<tr>
<td>$A$</td>
<td>4,117.0</td>
<td>13,586.0</td>
<td>15,877.0</td>
</tr>
<tr>
<td>$A / D$</td>
<td>1.25</td>
<td>1.20</td>
<td>1.10</td>
</tr>
</tbody>
</table>

Table 3.1. Collagen induces changes in FRET index ($F_C / A$) for aggregated DDR1. The symbols are as follows: $F_C / A$ is the FRET index described in the equation 4, $A$ is the acceptor intensity and $D$ is the donor intensity. The values shown above represent averages of multiple ROIs; standard deviation is given for the FRET index (statistical analysis of 20 ROIs per cell, 20 cells per sample and three separate experiments).
3.3.4. DDR1 aggregation occurs on the same time scale as the changes in the FRET index

Next we aimed to understand whether or not the increase of the FRET index upon collagen stimulation was related to the kinetics of DDR1 aggregation. For this purpose, 3T3 cells as used in the FRET studies, were transfected with the DDR1-YFP construct, and imaged live. As soon as 5 min after introducing collagen to the cells, DDR1 started to aggregate in a clustered distribution, as described in the previous sections. With increasing stimulation time – up to 30 min, this distribution pattern stays uniform for most of the cells present in the sample. After 60 min, the number of clusters decreases.

Quantitative analysis of the receptor distribution in the stimulated samples showed that the sharpest increase in receptor clustering occurs after 5 min, (30 ± 4.2% of DDR1-YFP is internalized) with only a mild increase (35.6 ± 3.6% at 30 min) thereafter. As shown in the Figure 3.5., the time scales of DDR1 aggregation and increase in FRET signal are similar. Evaluation of the average intensity on the entire cell showed no significant difference in the receptor population between the nonstimulated samples, and samples stimulated for various time intervals. This can be an indication that lysosomal degradation of the receptor does not occur in our experiments.
Figure 3.5. Dynamics of DDR1 aggregation. 3T3 cells transiently transfected with DDR1-YFP were imaged live before and after stimulation with collagen type I. (a) nonstimulated cells show uniform distribution of the receptor; (b) after 5 min and (c) after 30 min of collagen stimulation, DDR1 aggregates in clusters – indicated by white arrows; (d) quantitative analysis of the aggregation process (green, left axis) is depicted together with the increase in FRET (blue, right axis). The two processes are shown to have similar dynamics, with a sharp increase immediately following collagen stimulation and little variation thereafter.
3.3.5. DDR1 aggregation takes place through receptor internalization

Next we wanted to investigate the nature of collagen-induced DDR1 aggregation. For many RTKs, ligand binding results in internalization of the receptor. To test if this is the case for DDR1, we have used HEK 293 cells. These cells exhibit a thicker, less spread morphology, when plated on culture dishes that allows for microscopic localization on the plasma membrane of the fluorescently labeled receptors.

Transiently-transfected cells expressing DDR1-YFP were examined live, using widefield fluorescence microscopy. Prior to collagen stimulation, DDR1-YFP was found to be uniformly distributed on the cell membrane (Figure 3.6.a). Images of stimulated cells show aggregation of the receptor in clusters with a punctuate distribution (Figure 3.6.b). A dramatic loss of receptor population from the cell contour is observed for stimulated cells, indicating that the aggregated receptor is relocated from the plasma membrane into the cytoplasm. To confirm those results, similar samples were fixed and imaged using confocal microscopy. The resulting images (Figure 3.6.c) verify the membrane localization of the nonstimulated receptor. Collagen stimulated samples (Figure 3.6.d) showed that aggregated DDR1 was localized in the cytoplasm and not in the plasma membrane.

To further test that stimulated DDR1-YFP is undergoing cellular internalization, cells transfected with DDR1-YFP were stimulated with collagen, lysed using NP 40, a weak non-ionic detergent and directly probed for DDR1 expression using SDS PAGE and Western blotting.
**Figure 3.6.** DDR1 aggregation takes place through receptor internalization. HEK 293 cells were cultured on glass bottom culture dishes, transiently transfected with DDR1-YFP and stimulated with collagen type I for 10 min (as indicated); following stimulation, cells were imaged live using widefield fluorescence microscopy (a, b). Nonstimulated sample, (a) shows localization of the receptor on the cell membrane. (b) Stimulation sample shows loss of fluorescence from the membrane and aggregation of the receptors. Confocal imaging of fixed cells confirms localization of the receptor on the cell membrane in the nonstimulated sample (c) while stimulated cells (d) show a loss of receptor signal from the membrane accompanied by aggregation inside the cytoplasm. Nuclear staining is shown in blue. All scale bars are 10 μm.
The collagen-stimulated samples showed lower levels of DDR1 as compared with the nonstimulated samples (Figure 3.7.) indicating that smaller amounts of DDR1-YFP are present in the soluble fraction of the cell lysate after collagen stimulation. These results, along with our microscopic observations on the intact cell, (Figure 3.6.) indicate that aggregation of DDR1 takes place through internalization of the receptor and its incorporation in cytosolic vesicles (Richardson 2006).

![Figure 3.7.](image)

**Figure 3.7.** Collagen stimulation decreases the amount of soluble DDR in cell lysate. HEK 293 cells, transiently transfected with DDR1-YFP, were stimulated with 10 µg/ml collagen type I for 10 and 30 min intervals (as indicated). Cell lysates were electrophoresed using SDS PAGE and following Western blotting, the membranes were probed with anti-DDR1 antibodies (upper blot); DDR1-YFP is indicated by the horizontal arrow. A control untransfected sample – right most lane - does not show the YFP-tagged DDR1. The membranes were reprobed with anti-GAPDH antibodies to verify protein expression and loading (lower blot).
3.3.6. Internalized DDR1 is incorporated into the early endosome

To identify the cytosolic vesicles that incorporate DDR1 following its internalization, we have performed colocalization experiments with Rab5a, a marker for the early endosome (Nielsen 1999). In these experiments we have used COS 7 fibroblasts, were utilized since these cells are easily transfected and exhibit a well spread morphology that allows for accurate microscopic characterization of the fluorescent aggregates. Cells transiently co-transfected with DDR1-CFP and a GFP-tagged Rab5a expression construct, were fixed before or after collagen stimulation and examined by confocal microscopy. The perinuclear and nuclear regions generated nonspecific noise that could not be eliminated when imaging the entire cell. The samples that were not stimulated with collagen exhibited uniform distribution of both DDR1-CFP and Rab5a-GFP (Figure 3.8).

There was little overlap between the CFP and GFP channels, except for the noisy nuclear regions, indicative of lack of colocalization for the two proteins (Figure 3.8.c). In contrast, the stimulated samples demonstrate a punctuate distribution of DDR1-CFP and its reorganization into aggregates with granular or elongated morphology. The regions of aggregated DDR1 show a striking colocalization with Rab5a that generated aggregates of identical shape and size, at the same location (Figure 3.8.f). These results confirm that after interaction with collagen type I, DDR1 is internalized and incorporated into the early endosome.
**Figure 3.8.** DDR1 clusters colocalize with Rab5a. COS 7 cells were transiently transfected with both DDR1-CFP and Rab5a-GFP and stimulated with collagen type I for 30 min (as indicated). Confocal images of nonstimulated cells are shown on the left column, (a-c), and stimulated cells are shown in the right column, (d-f). CFP-green, GFP-red, and the overlap of the two—yellow, are shown in the same rows from top to bottom. (f) The clustered DDR1 – indicated by white arrows - in the stimulated cells is overlapping with Rab5a. The area of detail is the same for all images in the right column and indicated with the white rectangle in (d). All scale bars are 20 μm.
3.4. DISCUSSION

To study the oligomeric state of DDR1 we utilized FRET microscopy on intact cells. We found that the CFP- and YFP-labeled receptors exhibit close intermolecular interaction, indicative of dimer formation, even in the absence of collagen. This is consistent with recent reports by others (Noordeen 2006, Abdulhussein 2007) where DDR1 was shown to form ligand-independent dimers. Stimulation with collagen resulted in redistribution of a finite fraction (~30%) of the DDR1 population into aggregates possessing an elongated morphology. The FRET index increased significantly in the regions of aggregated receptors, from 5.4 ± 1.3 to 15.0 ± 2.6. Temporal characterization of DDR1 aggregation and FRET index variation showed the two processes to parallel each other, with a sharp increase immediately following collagen stimulation and little variation afterwards.

It is interesting to note that in both reports mentioned above, the cellular percentage of DDR1 dimers was found to be constant before or after collagen stimulation. Moreover, the reported percentage of dimers with respect to the overall receptor population was ~ 20%, similar to the percentage of DDR1 that we found to undergo aggregation during collagen stimulation ~ 30%. In our experimental set-up, the FRET index was linearly proportional with the percentage of acceptor-donor pairs. A transition from 30% to 100% dimeric percentage should result in a three time increase in the FRET index, which is what we detected in the regions of aggregated DDR1. Such a transition would be possible if collagen-induced aggregation applies only to DDR1.
dimers and not to DDR1 monomers. We propose that this is the case here, and that the detected increase in the FRET index is due to an increase in the percentage of DDR1 dimers in the regions of aggregated receptors. This interpretation is further supported by previous work from others showing that dimerization induces an increase in the affinity of DDR1 toward collagen type I (Leitinger 2003).

Further investigation of the collagen-induced aggregation of DDR1, revealed that this process was associated with a loss of fluorescence from the cell membrane and with a decrease in the receptor levels in the soluble part of the cell lysate. These results indicate that collagen induces relocalization of the receptor from the plasma membrane into the cytoplasm. Colocalization experiments with a marker of the early endosome, Rab5a, confirmed that following stimulation with collagen, DDR1 was internalized and incorporated into the early endosome. Receptor internalization upon ligand-induced activation is a commonly encountered cellular response for RTKs (LeRoy 2005, Sorkin 2007). Internalization can result either in degradation of the receptor and termination of the signaling event or can serve as a mechanism to localize the activated receptor at distinct locations in the cytoplasm.

Furthermore, the endosomal membrane is recently emerging as an important site of receptor initiated signal transduction (Sorkin 2007). Localization of the internalized receptor onto the endosomal membrane is reported to result in activation of signaling pathways that are distinct from those activated by the receptor incorporated into the cellular membrane (Kranenburg 1999). For
instance, mitogen-activated protein kinases (MAPK) signaling is activated by the Epidermal Growth Factor Receptor (EGFR) only after the receptor is internalized (Vieira 1996). The neurotrophin receptor TrkA, a RTK with the structure of the catalytic domain similar with that of DDR1, is reported to transiently activate Ras when present on the cell membrane, while endosomes containing activated TrkA-complexes, selectively mediate prolonged activation of Rap1 (Zweifel 2005, Wu 2001). It is not clear at this point if the internalized DDR1 is phosphorylated differentially and/or regulates a distinct signaling pathway, as compared with the receptor located in the cell membrane.

While collagen stimulation is reported to initiate DDR1 activation, the discrepancy between the large time interval required for full phosphorylation of the receptor ~ 90 min (Shrivastava 1997), and the small time interval ~ 5 min, the receptor spends on the cell membrane after collagen stimulation, suggests that other cellular processes are likely involved in DDR1 activation. One example in this direction is the reported inhibition of DDR1 phosphorylation by suppression of Src kinase activation (Dejmek 2003).

In conclusion the results of this study confirm our hypothesis, that the DDR1 oligomerization state mediates its interaction with collagen. Our findings here, corroborated with previous work by others, suggest that DDR1 dimerization, collagen binding and receptor phosphorylation are independent events, separated temporally and spatially from each other. We confirm that DDR1 receptors exhibit close intermolecular interactions, indicative of dimerization, in the absence of collagen and show for the first time that
collagen stimulation induces rapid internalization of the receptor at timescales prior to tyrosine phosphorylation. In addition, our results suggest that collagen-induced internalization applies preferentially to the dimeric DDR1 receptors. As the oligomerization state seems to designate different cellular pathways for DDR1, it remains to be seen what they are and how do they interrelate with each other. Further investigations along these lines may reveal if the collagen-induced aggregation of DDR1 consists of higher-order DDR1 oligomers, and what are the exact mechanisms for the delayed activation of the DDR1 receptor.
CHAPTER 4

DISCOIDIN DOMAIN RECEPTORS MODULATE FIBRILLOGENESIS OF COLLAGEN TYPE I

4.1. INTRODUCTION

Background

Extracellular collagen-binding proteins, as well as cell-surface collagen receptors like integrins, are reported to play a direct role in collagen fibrillogenesis. Integrins are the largest family of receptors that mediate cell adhesion to the ECM. Four integrin heterodimers are known to bind to collagen ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, $\alpha_{11}\beta_1$). Soluble $\alpha_1$ and $\alpha_2$ domains were shown to inhibit collagen fibrillogenesis \textit{in vitro} (Jokinen 2004). The kinetic dissociation constants were found to be higher for fibrillar vs. monomeric collagen type I in the case of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins. It was speculated that this constitutes the mechanistic explanation of integrin-supported pericellular fibril formation. In agreement with these findings, two reports indicate that collagen fibers formation is promoted by $\alpha_2\beta_1$ integrins (Velling 2002, Li 2003).

Among the soluble ECM proteins, the small leucine-rich proteoglycans (SLRPs) are thought to regulate collagenous matrix assembly in connective tissues (Linsenmayer 1990). The major proteoglycans in the corneal
stroma, lumican, keratocan, mimecan and decorin, are members of the SLRP family. Decorin contains a chondroitin/dermatan sulphate chain, a leucine-rich 45 kDa core protein, and is present in the ECM of numerous interstitial tissues (Sini 1997). Decorin binds type I collagen and it was shown to inhibit fibrillogenesis in vitro (Sini 1997). Moreover, the effects of decorin on collagen fiber formation in vivo were demonstrated to be tissue specific. Lumican-null mice were found to display alterations in collagen fiber diameter, as well as in collagen fiber density, which resulted in cloudy corneas (Danielson 1997). Fibronectin, a high molecular weight ECM glycoprotein, binds to collagen α1(I) chains at/or near the collagenase cleavage site. It has been established that fibronectin has a crucial role in fibrillogenesis of collagen type I and type III, and that integrins α1β1 and α2β1 strongly enhance this process (Johansson 1997).

**Hypothesis**

The effects of DDRs on collagen fibrillogenesis are not yet characterized. We showed that DDRs collagen-binding affinity is mediated by the fibrillar state of collagen (Chapter II) and that DDRs bind to overlapping collagen monomers (Agarwal, 2007). We propose that similar with other collagen receptors and collagen binding proteins, DDRs modulate fibrillogenesis of collagen type I. It is critical to understand if and how DDR binding can influence the collagen assembly process since the DDR ECD is present in several forms in vivo. If DDR binding to collagen were to influence collagen fibrillogenesis, then even the expression of kinase-dead isoforms of DDRs would be functionally significant,
as they may provide a novel mechanism for collagen regulation. We propose to study the effects of DDR binding on fibrillogenesis in vitro using purified DDR1-Fc and DDR2-Fc, for which the collagen-binding process was characterized in Chapter II of this dissertation. In addition, we will utilize cell-based assays wherein transient expression of DDR1 or DDR2 will be induced using transfection with plasmids encoding the entire mouse DDR sequence.

4.2. MATERIALS AND METHODS

4.2.1. Materials

FITC-conjugated collagen type I, which is a highly purified telopeptide free extract from bovine skin was purchased from Sigma Aldrich (St. Louis. MO). DDR1-and DDR2-myc plasmids encoding the entire mouse DDR1 or DDR2 tagged with triple myc-tag as well as purified DDR1-and DDR2–Fc proteins were from Regeneron Pharmaceuticals (Tarrytown, NY). Purified acid solubilized collagen type I was purchased from Vitrogen - Cohesion Technologies Inc. (Palo Alto, CA).

4.2.2. Turbidity measurements

Monomeric collagen was diluted (5 to 50 μg/ml) in PBS at a pH of 7.2 on ice. A UV-visible spectrophotometer (UV-2401 PC, Shimadzu, Columbia, MD) equipped with a temperature control chamber and a multiple cuvette assembly was used for collagen turbidity measurements. Due to the high amounts of DDR protein required for these experiments, the majority of
measurements were performed using 15 µg/ml collagen, a concentration that is above the reported critical concentration required for fibrilization at 37°C (Na 1989, Kadler 1987). Nevertheless, trial runs were performed using a collagen concentration of 50 µg/ml (data not shown) with similar results. Collagen fibers formation was estimated from turbidity measurements, by measuring the absorbance at 313 nm as a function of time. The collagen samples were maintained at 37°C in the spectrophotometer, conditions under which collagen is known to fibrillize (Kadler 1996, Jokinen 2004). Four identical 1 ml quartz cuvettes were used in each experiment and a baseline for each cuvette was recorded using PBS. Thereafter, the cuvettes contained 50 nM collagen type I (15 µg/ml) in either PBS alone, or with oligomers of TrkB-Fc, DDR1-Fc or DDR2-Fc in identical concentrations. The kinetics of fiber formation was recorded by measuring the absorbance every 60 seconds. The final concentration of DDR-Fc in oligomeric solutions ranged from 1 to 5 µg/ml (3 to 14 nM) in DDR-collagen mixtures. Experiments were recorded continuously for 8-10 hrs, and the samples were preserved thereafter for microscopic analysis.

4.2.3. Atomic Force Microscopy

Freshly cleaved mica (Ruby muscovite, S & J Trading, Glen Oaks, NY) was used as a substrate for all AFM samples. Samples from the turbidity experiments described above were collected and centrifuged at 14,000 g for 30 min. The supernatant was collected and used for AFM studies after a dilution of 1:5 in filtered distilled water. An aliquot (20 µl) of each sample was spread on
mica and incubated for 10-15 min. The samples were then blotted, air-dried and imaged in ambient air.

A Multimode AFM with a Nanoscope IIIa controller (Digital Instruments, Santa Barbara, CA) was used in tapping mode for AFM imaging. For fluid imaging, samples were imaged in filtered, distilled water or in filtered PBS using a fluid cell. Silicon nitride cantilevers of the type NP-20 (Digital Instruments) with a nominal spring constant of 0.32 N/m were driven at a resonance frequency of ~ 9 kHz in an aqueous medium. For imaging in air, NSC-15 silicon cantilevers (MikroMasch, Estonia) were used at a resonance frequency of ~ 320 kHz. The vertical engage scanner of type ‘JV’ was used; the scanning rate was 2 Hz with 512 lines per scan direction. Images were recorded in height and amplitude modes with scan sizes ranging from 1 to 30 µ².

For AFM data analysis, height measurements of the DDR proteins were accomplished using AFM height images. For this purpose, the AFM images were ‘flattened’ and used with no further processing. The vertical ‘z’ height was estimated by drawing a line across the image and measuring the height of the ‘peaks’ with respect to the mica baseline by using the section-analysis feature of the Nanoscope software (v5.32r). For collagen length measurements, the ImageJ software (NIH) was used on tif files of AFM height and amplitude images. The collagen contour was manually traced onto the image and the length ascertained using ImageJ. For the length and height analysis, n=50 points were collected from at least three different images from the same or identically prepared samples.
4.2.4. Transmission Electron Microscopy

To examine the morphology of collagen fibers formed in the presence of DDRs-Fc, samples from the turbidity experiments were centrifuged at 14,000 g for 30 min. The supernatant was removed and the pellet re-suspended in 20 μl of fresh PBS. An aliquot of the suspension was spotted on 400 mesh formvar-carbon coated grids (Electron Microscopy Sciences, Hatfield, PA), left for 5 min, blotted, washed twice with distilled water and stained with 0.5% uranyl acetate for 60 sec. The grids were finally blotted, air dried and examined using a Zeiss EM 900 transmission electron microscope (TEM) at 80 kV. Grid squares containing collagen fibers were selected and images were recorded at magnifications ranging from 7 K to 50 K using a Mega View III digital camera (Soft-Imaging, Lakewood, CO) coupled to the AnalySIS software (Soft-Imaging).

TEM data analysis was accomplished using the AnalySIS software on images recorded at a magnification of 30 K to 50 K. The diameter and periodicity of collagen fibers were analyzed for n=100 fibers selected from at least three independent and identical experiments. As far as possible, overlapping or intertwined regions of fibers were excluded from the analysis. Fiber diameters were measured near the central ‘trunk’ of the fibers, away from their tapering conical ends. The periodicity of collagen fibers was determined by measuring the thickness of the light and dark bands along the fiber contour. The fibers were further classified as possessing a banded or a non-banded structure.
4.2.5. Hydroxyproline assay

To measure the total quantity of fibrillar collagen formed in the presence or absence of DDRs-Fc, we used a chromatographic method (Reddy 1996, Huszar 1980). Briefly, 1 ml samples containing 50 μg/ml collagen were incubated for 17 hrs at 37°C in the presence or absence of DDR-Fc or TrkB-Fc oligomers at a final concentration of 10 μg/ml. This ratio of DDR-Fc oligomers to collagen (1:5) was similar to the ratio used in the turbidity measurements described earlier. To separate the fibrillar collagen, samples were centrifuged for 5 min at 14,000 g as reported by others (Kuznetsova 1998, Salchert 2004). The supernatants were preserved and the pellet was resuspended in a 50 μl volume in presence of 4N NaOH and used for the hydroxyproline assay.

The samples were hydrolyzed by autoclaving at 120°C for 15 min. Oxidation of the hydrolyzate was accomplished by addition of 450 μl of Chloramine-T reagent followed by 25 min incubation at room temperature. Following oxidation, 500 μl of aldehyde/perchloric acid solution was added and the mixture was incubated 20 min at 65°C for chromatophore development. Absorbance was then measured at 563 nm in a Beckman – Coulter DP 600 spectrophotometer. In parallel, standards were prepared using dried hydroxyproline resuspended in water in the range of 0 to 2 μg. Further, in order to estimate the actual collagen content in our pellet samples, we used a set of collagen standards containing cold, monomeric collagen in the range of 0 to 50 μg (50 μl total volume).
4.2.6. Cell culture and Fluorescence Microscopy

Mouse osteoblast MC3T3-E1 subclone 4 cells obtained from ATCC were cultured in Minimal Essential Medium Alpha (MEM alpha) from Gibco, supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (pen-G 10,000 units/ml; streptomycin 10,000 µg/ml; amphotericin-B 25 µg/ml). Cells were seeded on 25 mm glass coverslips and transiently transfected with DDR1-myc or DDR2-myc using Fugene 6 (Roche, Switzerland). After 48 hrs the media was aspirated and replaced with fresh media containing FITC-conjugated collagen at a final concentration of 1 µg/ml, and the culture dishes containing the coverslips were placed back in the incubator for time intervals of 1, 3 and 6 hrs. This collagen concentration was selected after incubating samples containing from 1 to 5 µg/ml FITC-labeled collagen at 37°C, to determine the concentration at which collagen does not assemble into fibers, independent of cells. At the end of the incubation period, the coverslips were washed three times with PBS and fixed with 2% paraformaldehyde in PBS for 30 min. Following the fixation protocol the cells were nuclear stained using 4 µM bisbenzimide (Calbiochem) and the coverslips mounted on microscope glass slides using Prolong mounting media (Invitrogen). Control samples were prepared incubating collagen on dishes containing only glass coverslips. Also, samples containing cells without collagen were prepared in order to check for background fluorescence in the FITC acquisition channel.

To test the efficiency of the transfection protocol, similar cultures were lysed and the protein content was electrophoresed using SDS PAGE as
described in Chapter III (3.2.4). Following electrophoresis, the proteins were transferred on nitrocellulose membranes and probed with mouse monoclonal anti-myc antibodies (Cell Signaling Technologies, Boston MA).

Microscopy was performed using a confocal Zeiss LSM 510. The bisbenzimide stained nuclei were visualized using a tunable Ti-Sapphire laser at 750 nm in two-photon mode. The FITC channel was excited using the 488 nm line of the Argon laser. The images were acquired using both 20X and 63X objectives at an acquisition rate of 12 kHz with 512 lines per scan direction.

4.3. RESULTS
4.3.1. DDRs modulate fibrillogenesis of collagen type I

To investigate the role of DDRs in collagen fibrillogenesis, collagen turbidity measurements were performed in the presence or absence of DDRs-Fc. Having established that both DDR1-Fc and DDR2-Fc in the oligomeric state exhibit enhanced binding to collagen, these experiments were performed using DDR-Fc oligomers. As shown in Figure 4.1., the turbidity (absorbance) increases with time as expected during collagen fibrillogenesis. Two features may contribute to the increase in turbidity: (a) the assembly of monomeric collagen into fibrils and fibers, and (b) binding of DDRs-Fc oligomers to collagen. To rule out the second possibility, collagen turbidity experiments were also conducted at 18°C, since collagen does not form fibers at this temperature, but does bind DDR. In these experiments, no increase in signal was observed, and all four cuvettes exhibited near-zero parallel curves (data not shown). Therefore
the increase in signal observed in our turbidity measurements is primarily due to the collagen fibers formation process.

Figure 4.1.a shows the results of a turbidity measurement experiment, where the concentration of DDRs-Fc in oligomeric form was 3 μg/ml (5 nM) and the collagen concentration was 15 μg/ml (50 nM). In the sample with collagen alone (PBS), absorbance increases rapidly, reaches a noisy region around 1 hr and then saturates. Such a turbidity curve is characteristic of collagen type I fibrillogenesis (Kadler 1996, Jokinen 2004). The time interval that precedes the increase in signal is termed lag time ($t_{\text{lag}}$) in literature. The samples with TrkB-Fc oligomers showed a similar feature except that the sharp rise in absorbance is delayed by a $t_{\text{lag}}$ of about 2 hrs. This delay is likely due to steric hindrance exercised by TrkB-Fc oligomers onto the self-assembly of collagen molecules. In samples with DDR1-Fc, $t_{\text{lag}}$ is around 2.4 hrs, after which the absorbance rises sharply and reaches a steady state higher than that of the other three samples. Samples containing DDR2-Fc oligomers exhibit a $t_{\text{lag}}$ of over 4 hrs. Such a prolonged plateau region is not prominent in samples containing TrkB-Fc or DDR1-Fc. The rate of increase in signal thereafter is also more gradual for DDR2-Fc. In a separate series of experiments, increasing the concentration of DDRs-Fc significantly increases their $t_{\text{lag}}$ as shown in Figure 4.1.b.
Figure 4.1. Turbidity measurement of collagen fibrillogenesis. (a) Collagen fiber formation was recorded by measuring turbidity of collagen solution at 37°C as function of time. Addition of TrkB-Fc, DDR1-Fc, DDR2-Fc oligomers (3 μg/ml) is indicated by arrows; PBS denotes the sample containing only collagen. The lag time ($t_{lag}$), after which absorbance rises sharply was highest for DDR2-Fc; samples with DDR1-Fc oligomers have the highest absorbance values after completion of fibrillogenesis. (b) Effect of DDR1-Fc, DDR2-Fc and TrkB-Fc concentration on $t_{lag}$. 
DDR2-Fc was found to have a stronger inhibitory effect on collagen fibrillogenesis as compared to DDR1-Fc or TrkB-Fc. This is indicated both by the larger \( t_{\text{lag}} \) and by the lower ‘noise’ level observed in samples containing DDR2-Fc. To compare the amount of fibrillar collagen formed in the presence of DDRs-Fc with that formed in samples containing collagen alone or TrkB-Fc proteins, similar samples with 50 µg/ml collagen were incubated overnight at 37º C, centrifuged and the collagen content of the pellet measured using the hydroxyproline assay.

Our results showed that nearly 50-55% of collagen was incorporated into the fibrillar form (obtained as pellet) and no significant difference was observed in the amount of fibrillar collagen in all four samples (Figure 4.2.). Also, samples containing either collagen alone or collagen in the presence of DDR2-Fc oligomers were incubated for a period of 1 hr - less than the lag time of collagen fibrilization in the presence of DDR2-Fc - at 37º C. After incubation, the samples were processed as above, and the fibrillar collagen content measured with the hydroxyproline assay. The amount of fibrillar collagen was much smaller in samples containing DDR2-Fc compared with samples containing collagen alone, or collagen plus either DDR1-Fc or TrkB-Fc. This was in agreement with the results of the turbidity measurement experiments that indicated DDR2-Fc delays formation of collagen fibers.
4.3.2. Effect of DDRs on Collagen Morphology

To assess the collagen morphology in the presence of DDRs-Fc, samples from turbidity measurement experiments were centrifuged and the supernatant examined using AFM in ambient air. Several different regions on each sample were imaged, and a representative region is presented in Figure 4.3. The collagen morphology in samples with DDR2-Fc was strikingly different from that of collagen in samples containing TrkB-Fc or DDR1-Fc.
Quantitative analysis of collagen length (n=50) from AFM images (inset, Figure 4.3.) revealed that samples with DDR2-Fc had collagen predominantly in monomeric state (< 300 nm in length). The other three samples had collagen in dimeric state (300 to 600 nm in length) or longer collagen fibrils. In contrast, the samples with DDR1-Fc were most abundant in longer fibrils. The overall collagen morphology in samples containing TrkB-Fc or collagen alone looked similar, consisting mainly of collagen dimers and longer fibrils. The AFM images of TrkB-containing samples exhibited a large population of particulate material that likely represents the unbound TrkB-Fc oligomers present in the supernatant.

To further examine the morphology of collagen fibers and the matrix formed in the presence of DDRs-Fc, the pellet from the turbidity experiments samples was preserved after centrifugation and examined using TEM. Figure 4.4. shows representative TEM images from all four samples at magnifications ranging from 7 K to 50 K. No apparent difference in fiber morphology is visible at a low magnification of 7 K in all four samples. However, at higher magnifications (30 K to 50 K), the samples with collagen alone showed a mixture of banded collagen fibers along with some incompletely formed fibers. The D-periodicity in collagen fibers formed in samples with collagen alone or with TrkB-Fc was found to be 68 ± 2 nm as determined from TEM images. The samples with DDR1-Fc and DDR2-Fc showed a very weak or a complete lack of periodicity in collagen fibers, as compared with collagen alone or with TrkB-containing samples.
Figure 4.3. AFM assessment of collagen fibers. Amplitude images of collagen present in the supernatant formed after centrifugation of samples incubated overnight. A quantitative analysis of collagen length for n=50 filaments for each sample is shown as inset (center). Collagen in the presence of TrkB-Fc, PBS (collagen alone) and DDR1-Fc forms dimers (300-600 nm in length) and long fibrils (> 600 nm) with the number of long fibrils being highest for the DDR1-Fc sample. In contrast collagen in presence of DDR2-Fc is mostly in the monomeric (< 300 nm) state.
Quantitative measurements of collagen fibers diameters (Figure 4.5.) revealed important differences between samples incubated with the DDRs-Fc and those incubated with TrkB-Fc or collagen alone. While the average fiber diameter for samples with collagen alone, TrkB-Fc and DDR2-Fc ranged from 64 to 72 nm, the samples with DDR1-Fc had thicker collagen fibers with an average diameter of 93 nm and a more scattered distribution. Along with the diameter measurements, each fiber was classified as having or not having a banded structure.

Our analysis shows that while 60% to 70% of the fibers were banded in TrkB or collagen alone samples, only 10% of the fibers were banded in the samples containing DDR2-Fc and 30% of the fibers were banded in the samples containing DDR1-Fc. Further, the banded structure, when present for DDRs-Fc containing samples, was often significantly weak in contrast or discontinued.
Figure 4.4. TEM assessment of collagen fibers. TEM images of collagen fibers formed with and without DDRs. At low magnification (7 K, upper row), no apparent difference in fiber morphology is observed between the various samples. The periodic banded structure of collagen fibers in samples with collagen alone (PBS) or TrkB-Fc can easily be observed at magnifications of 30 K or 50 K (middle and lower rows). Fibers formed in the presence of DDRs-Fc lack the periodic banded
Figure 4.5. Distribution of collagen fiber diameters. We measured $n=100$ fibers in the TEM images at a magnification ranging from 30 to 50 K. The fiber diameters ($D$) have been binned into 5 nm bins. The average fiber diameter and standard deviation are indicated for each sample. Further, each fiber was classified for presence or absence of banded structure and the number of banded fibers ($B$) in each bin is represented in red.
4.3.3. Overexpression of DDRs inhibits collagen fibrillogenesis in cell-based assays

To examine how cellular expression of DDRs affects collagen fibrillogenesis, 3T3 mouse osteoblast were cultured on glass coverslips and transiently transfected with full length DDR1-myc or DDR2-myc. Expression of DDRs-myc was verified using Western blotting (Figure 4.6.). Following transfection (48 hrs), native and DDR-transfected cells were incubated with FITC-labeled collagen type I (1 μg/ml) for 0 to 6 hrs and analyzed for collagen fibers formation using laser scanning confocal microscopy (LSM). As a control, samples containing cells without collagen or collagen without cells were examined under identical conditions. No background fluorescence was observed on the cell samples (Figure 4.6.). Collagen alone did not show any detectable fluorescence when incubated for 1 or 3 hrs; when incubated for 6 hrs, samples with collagen but not cells, exhibited small fluorescent specs (Figure 4.6.), which were negligible compared with the corresponding samples with cells.

We observed a striking difference in the morphology and quantity of collagen fibers formed in the presence of native, untransfected cells compared with those formed in the presence of DDR-transfected cells. Collagen in the untransfected cells samples followed a polymerization process, where short, thin fibrils could be detected after about 1 hr (Figure 4.6.c). With increasing incubation times, collagen fibers were observed to become longer and thicker (Figure 4.6.d, e). In contrast, collagen formations observed in DDR-transfected samples did not resemble fibrillar morphology.
Figure 4.6. DDRs inhibit collagen fibrillogenesis on the cell surface. 3T3 cells nontransfected (native) or transiently transfected with either DDR1 or DDR2-myc were used; Transfection of DDRs-myc was verified using Western blotting. (a) LSM images of native cells, without collagen show no fluorescence in the FITC channel. Nuclear staining is shown in blue. (b) Collagen without cells shows negligible fluorescence even after 6 hrs of incubation. (c) to (e) Collagen fibers formation in the presence of native cells after 1, 3 and 6 hrs of incubation. (f) to (k) Collagen aggregates developed on cells overexpressing DDRs-myc for the same time intervals with little or no collagen fibril assembly. All scale bars are 50 µm.
After 1 hr, instead of thin, short fibrils the DDRs samples exhibited a few large clusters or aggregates of collagen which had a granular appearance (Figure 4.6.f, k). With increasing incubation times of 3 and 6 hrs, these collagen aggregates remained almost the same in size exhibiting an increase in intensity (possible indication of increased collagen incorporation). Overall, the DDRs-myc-transfected cells showed little or no evidence of collagen fibers formation compared with native cells.

4.4. DISCUSSION

The studies described here confirmed our hypothesis that DDR receptors modulate collagen fibrillogenesis. In our in vitro experiments, both DDR1 and DDR2 were found to modulate fibrillogenesis of collagen type I, although in different manners, suggestive of their different binding sites and/or mechanisms. DDR2 was found to be a strong inhibitor of collagen fibrillogenesis as demonstrated by both the long $t_{\text{lag}}$ in turbidity measurements and the observation that the collagen supernatant consisted largely of monomeric collagen. Further, the collagen fibers formed in presence of DDR2 exhibited a striking lack of the periodic banded structure, which is characteristic to native fibers. Our earlier investigations have revealed that DDR2 can bind at three sites on the collagen molecule (Agarwal 2002). Thus, it is likely that the DDR2 binding site on collagen plays an essential role in the natural self-assembly of collagen molecules and in forming native, banded fibers.

In contrast, the effect of DDR1 on collagen fibrillogenesis was
very different from that of DDR2. The most striking feature with DDR1 was the sharp rise in turbidity after a smaller $t_{lag}$ followed by a much higher overall absorbance. The higher absorbance value suggests formation of collagen fibers with larger diameters (Ge 2004). And indeed, this thickening of collagen fibers in the presence of DDR1 was confirmed by direct observation in our TEM studies. In addition, the supernatant from collagen incubated with DDR1 consisted largely of collagen dimers and longer collagen fibrils. These observations lead us to speculate that DDR1 promotes fibrillogenesis of collagen. However, the fact that the native banded structure of collagen fibers was disrupted, suggests that the rapid rate of fiber formation in the presence of DDR1, disrupts the banded structure of collagen fibers.

The results of the hydroxyproline assay showed that equal amount of fibrillar collagen was formed in the presence or absence of DDRs after fibrillogenesis reached equilibrium (overnight incubation). Thus, although DDRs delay the collagen fibrillogenesis process and alter the morphology of collagen fibers, they do not alter the net amount of collagen that is incorporated in fibrillar form over extended periods of time.

Our results indicate that weakly or non-interacting proteins like TrkB delay collagen fibrillogenesis. This can be explained using the model proposed by Parkinson et al. (Parkinson 1994) where fibrillogenesis can be modeled as diffusion-limited aggregation of individual collagen molecules. The presence of other proteins is likely to provide steric hindrance and thus limit the rate of diffusion of collagen monomers, thus leading to delay of fibrillogenesis. As
expected, the diameter and banded structure of collagen fibers formed in the presence of TrkB was identical to that of collagen alone.

A similar delay in collagen fibrillogenesis and altered collagen morphology was observed in our cell-based assays, which signifies that DDRs play a role in collagen fibers formation in vivo. Both DDR1 and DDR2 inhibited collagen fibrillogenesis at the cellular level, promoting formation of large aggregates of collagen instead of collagen fibers. These collagen aggregates exhibited a granular instead of fibrillar morphology at the light microscopy level, suggesting that DDRs may be inhibiting collagen fibrillogenesis by “locking” the collagen molecules in monomeric state. The aggregates formed on DDR1-overexpressing cells were initially more abundant and larger in size compared with the aggregates formed on DDR2-overexpressing cells. This may be explained by a rapid cross-linking of collagen molecules by DDR1, as has been observed in our in vitro experiments (giving rise to maximum turbidity readings for DDR1 samples). The size of the collagen aggregates diminished with time, more rapidly for the DDR1-transfected cells, as compared with the DDR2-transfected cells. A possible cause for this may be the shedding of DDR1 ECD from the cell surface upon interaction with collagen.

It is interesting to note that the effect of DDRs expression on collagen fibrillogenesis in our cell-based assay was observed for a collagen concentration much below 10 μg/ml, optimally required for DDR activation (Vogel 1997, Shrivastava 1997) and as early as one hr after addition of collagen, which is below the optimal time (90 min) required for activation of DDRs by
collagen (Vogel 1997). It has been reported that activation of DDRs can lead to upregulation of matrix metalloproteinases (MMPs) but these are detectable only after prolonged collagen stimulation of 1 to 4 days (Vogel 1997). Therefore, it is unlikely that the altered collagen morphology on DDRs-overexpressing cells is due to the cleaving action of MMPs.

The cells used in these assays, 3T3 mouse osteoblast, endogenously express low levels of DDR1 and most likely DDR2; however, inhibition of fibrillogenesis was not observed for the nontransfected cells. The implication is that overexpression of DDRs is needed for this effect, situation encountered in vivo, where DDRs are upregulated in a number of malignancies and pathologies. It would be interesting to compare if knockdown of DDRs leads to enhanced collagen deposition. Recent reports have demonstrated increased collagen deposition in DDR1 knock-out mice (Franco 2008). Obviously, the in vivo mechanisms of collagen regulation are more complex, and additional factors could play important roles.
CHAPTER 5

CONCLUSIONS AND DISCUSSIONS

Here we give a summary of the main results from the previous chapters, followed by a schematic representation of their implications on the mechanism of collagen regulation by DDR1. We analyze the biological significance of our results and propose further directions of research to test their implications.

The oligomeric state of DDR1 mediates its interaction with collagen

We have quantitatively characterized the role of DDR1 oligomeric state in its interaction with collagen, using SPR and FRET microscopy experiments. We established that antibody-mediated oligomerization of dimeric DDR1-Fc and DDR2-Fc significantly enhances their binding to immobilized collagen type I in vitro, as demonstrated by SPR experiments. Reports by others, utilizing different fusion constructs, showed that dimerization alone of both DDR1 and DDR2 is necessary and sufficient for binding to collagen (Leitinger 2003). It is possible that differences in the constructs used, resulted in a different relative orientation of the dimer’s monomeric components. This indicates that collagen binding to DDRs requires a specific dimer structure.
Further, using FRET microscopy on live cells, we have found that DDR1 forms dimers in the absence of collagen. This is consistent with recent reports by others (Noordeen 2006, Abdulhussein 2007) where DDR1 was shown to form ligand-independent dimers. FRET microscopy demonstrated that the DDR1 dimers are uniformly distributed over the entire cell surface. Collagen stimulation induced rapid aggregation of DDR1 receptors at time scales below its reported activation interval. Quantification of this process, together with our FRET measurements, lead us to speculate that collagen-induced aggregation applies only to DDR1 dimers and not to DDR1 monomers. This is consistent with previous work by our and other groups, showing that DDR1 dimers exhibit higher affinity toward collagen, as compared with DDR1 monomers.

**Collagen induces internalization of DDR1**

Using confocal microscopy and biochemical analysis, we demonstrated that collagen induces internalization of the aggregated DDR1. Colocalization experiments with an endosomal marker showed that following internalization the receptor is incorporated into the early endosome.

This is the first time that internalization of DDR receptors has been reported, however, receptor internalization upon ligand-induced activation is a common cellular response for RTKs (LeRoy 2005, Sorkin 2007). Internalization can result either in degradation of the receptor and termination of the signaling event, or it can serve as a mechanism to localize the activated receptor at distinct locations in the cytoplasm. Our measurements showed no significant
difference in receptor levels between nonstimulated and collagen-stimulated samples. This can be an indication that lysosomal degradation of the receptor did not take place in our experiments.

**Collagen fibrillar state mediates DDRs binding**

We utilized SPR to test whether DDRs recognize the fibrillar state of collagen. The dissociation constants of DDR1 and DDR2 oligomers were nearly identical suggesting that they have an equal collagen-binding affinity. The SPR experiments demonstrated that both DDR1 and DDR2 have reduced affinity toward fibrillar collagen, as compared with monomeric collagen. These results can have significant physiological relevance, especially in regions where monomeric-collagen expression is increased, and active ECM remodeling occurs, such as in wound healing, vascular disorders or cancer metastasis.

**DDRs modulate fibrillogenesis of collagen type I**

Our observations that DDRs affinity toward collagen is mediated by the fibrillar state of collagen, and that DDRs bind to overlapping collagen monomers (Agarwal, 2007), along with the demonstrated tendency of collagen-binding proteins to regulate collagen fibrillogenesis, led us to investigate whether DDRs have a role in collagen fibrillogenesis. Indeed, using both *in vitro* and cell-based assays, we have shown that both DDR1 and DDR2 modulate fibrillogenesis of collagen type I. The collagen fibers formed *in vitro* in the presence of DDRs ECD exhibit lack of banding, which is an indicative of
disrupted fiber structure. Also, the diameter of the collagen fibers formed in the presence of DDR1 ECD was significantly larger than that of native fibers. DDR2 ECD was found to significantly delay the fibrillogenesis process. Our cell-based assays demonstrated that overexpression of both DDR1 and DDR2, inhibits formation of collagen fibers, at timescales that indicate the process is independent of MMP upregulation.

**Model of Collagen regulation by DDR1**

Currently, DDRs are known to regulate collagen in two ways. It is well established that DDRs activation results in upregulation of matrix metalloproteinases (MMP) that cleave the collagen fibers in ECM. Secondly the activation of DDRs results in increased expression of collagen α-chains. However, both these processes take place after prolonged (~24 hr) activation of DDRs.

Based on the new results presented in this dissertation with regard to the rapid collagen-induced internalization of DDR1, the role of DDR1 oligomerization, the recognition of collagen fibrillar state by DDRs and the modulation of collagen fibrillogenesis by DDRs, we propose the following model of collagen regulation by DDR1 (Diagram 5.1).
Diagram 5.1. Collagen regulation by DDR1. DDR1 exists on the cell membrane as a mixture of monomers and dimers. High-affinity interaction between DDR1 and collagen monomers induces internalization of DDR1 dimers and activation of the endosomal signaling pathway; Endosome signaling induces DDR1 activation followed by recycling of DDR1 dimers to the cell surface. DDR1 signaling results in MMP upregulation, shedding of the DDR1 ECD into the ECM (where it can directly interfere with collagen fibrillogenesis), and upregulation of collagen synthesis.
We propose that high-affinity interaction between monomeric collagen and DDR1 receptors induces internalization of DDR1 dimers and their incorporation into the early endosome. Endosomal membrane mediated signaling, induces DDR1 activation, followed by recycling of the internalized DDR1 dimers to the cell membrane. We propose that DDR1 internalization is a key regulatory step in the activation mechanism of this receptor. DDR1 activation results in MMP upregulation, shedding of DDR1 ECD and upregulation of collagen synthesis. All three processes enhance ECM remodeling by cleavage of the collagen fibers, regulation of fibrillogenesis and upregulation of collagen synthesis. In parallel to regulating DDR1 activation, internalization of DDR1 dimers should have direct consequences on cell adhesion, as it results in a diminished number of collagen-binding sites present on the cell surface. Moreover, the DDR1 monomers that remain on the cell surface have a lower affinity for collagen as compared with the internalized DDR1 dimers.

Further work is required to test this model of collagen regulation. An important question that has to be answered refers to the fibrillar state of collagen required for initiation of DDR1 internalization. In our studies of DDR1 aggregation and internalization, the collagen was most likely a mixture of monomeric and fibrillar collagen. Preliminary experiments conducted at 4°C, where the collagen does not fibrilize, showed the same rate of DDR1 aggregation. This suggests that monomeric collagen induces aggregation and internalization of DDR1, an observation that is supported by the demonstrated high-affinity interaction between DDR1 receptors and monomeric collagen.
However, at this stage we do not know whether fibrillar collagen also induces DDR1 aggregation. The next step would require stimulation of DDR1 with fibrillar collagen in the absence of monomeric collagen. This was not possible to achieve in our current experimental set-up; future experiments would require coating of cell culture substrates with fibrillar collagen.

To better understand the role of internalization in DDR1 activation, a more detailed characterization of the process is needed. Following ligand-induced activation, many RTKs are internalized through the clathrin-coated pit pathway (Wiedlocha 2004). Dynasore, a recently discovered chemical inhibitor of this internalization pathway, was used successfully in a series of studies of receptor internalization (Kirchhausen 2008, Nankoe 2006). Here dynasore could be used to probe whether DDR1 internalization takes place through the clathrin-coated-pit pathway and if successful, it can be incorporated in a line of experiments designed to separate the membrane mediated DDR1 signaling from the endosome mediated DDR1 signaling. Along the same line of investigation, an important question to be answered addresses the coexistence of activated DDR1 monomers and dimers on the cell surface. Imaging based experiments offer the ability to simultaneously probe for activation/phosphorylation and dimerization, and at the same time to generate topographical information.

Preliminary results showed that internalized DDR1 does not undergo lysosomal degradation, as indicated by constant receptor levels. However, further work is needed to answer this question. Colocalization experiments with lysosomal markers should be carried out to probe for
incorporation of internalized DDR1 into the lysosomal compartments. DDR1 internalization has the potential to mediate the intracellular degradation of collagen. As previously described (Chapter 1.2.2), this is a well established route of collagen turnover for other membrane-bound collagen receptors. Preliminary results in our laboratory showed that this is the case for DDR1 also; however, further work is needed in order to characterize this process.

Our studies of the DDR oligomeric state and cellular distribution were confined to DDR1. There is even less known about the oligomeric state of DDR2 on the cell membrane, and about the effects of collagen on DDR2 cellular distribution and oligomerization. Studies similar with those presented here could be used to obtain an answer to these questions.

An alternative route of fibrillar collagen regulation in ECM takes place through DDRs ECD. As mentioned earlier, in addition to their incorporation in the full length DDR1 and DDR2, the expression of DDR ECD can be regulated via two mechanisms: (1) in DDR1 splice variants (Alves 2001) and (2) proteolytic cleavage and shedding of DDR1 ECD (Vogel 2002). As depicted in Diagram 5.1, activation of DDR1 results in shedding of the DDR1 ECD into the ECM, where it can directly interfere with collagen fibrillogenesis, and generate improperly formed fibers that lack D periodicity. Parallel research in our laboratory demonstrated that expression of a membrane-anchored, kinase-dead DDR2 protein (that contains DDR2 ECD) in stable cell lines, delays the kinetics of collagen fibrillogenesis and results in fibers with altered morphology (manuscript submitted). A similar role can be played by the kinase-dead
isoforms of DDR1. Our results signify the importance of identifying and characterizing the DDR2 isoforms that contain the DDR2 ECD.

As a major component of ECM, collagen fibers play an important role in defining ECM's mechanical properties. An interesting question is whether the observed disruption of the collagen fibers structure by membrane-anchored and soluble DDR ECD results in modifications of the fiber's mechanical characteristics. This is an important question, as it has been shown that collagen fibers are subjected to mechanical stress by fibroblasts, through cell-surface integrins (Lee 1999). Also, recent studies have demonstrated that the rate of fibroblasts displacement depends on the stiffness of the matrix in which they move (Freyman 2002). Ongoing research in our laboratory is pursuing the characterization of collagen fiber's mechanical properties. Preliminary results show that DDRs modulate the mechanical properties of collagen.

**Physiological ramifications**

Our results support the role of DDR receptors as potent regulators of collagen deposition in ECM. As such, they play an important role in modeling/remodeling of the connective tissue, during embryonic development and adult phase. By binding to collagen they enhance the cell adhesion to ECM, and the internalization/recycling loop we demonstrate here, can serve as a modulator of cell migration and proliferation. As described in the previous section, both DDR1 and DDR2 have the potential to regulate the mechanical properties of ECM, with direct effects on cell migration.
Indeed, mice lacking either DDR1 or DDR2 exhibit dwarfism, which in the case of DDR2-null mice was linked to reduced proliferation of chondrocytes (Vogel 2006). Also, aberrant collagen deposition in knock-out animals revealed that DDR receptors play an important role in development and functioning of several organs, including the mammary gland, the vasculature and the kidney. Normal physiological processes that are associated with increased ECM remodeling and/or collagen deposition, such as embryo implantation in mice, were reported to be inhibited in DDR1-null mice (Vogel 2001). The process of wound healing, during which collagen synthesis is upregulated and ECM undergoes extensive remodeling, is reported to be inhibited in DDR2-null mice.

The elevated DDRs expression in a number of invasive tumors was suggested to play an important role in tumor proliferation and stromal invasion. Our results suggest that overexpression of DDRs may enhance ECM degradation through inhibition of collagen fibrillogenesis and upregulation of MMP. DDR receptors have been associated with atherosclerosis and fibrosis of kidney, liver, lung and skin, diseases where regulation of collagen content plays an important role. However, as DDRs are directly involved in regulation of collagen synthesis, proliferation of collagen-producing fibroblasts and in degradation of collagen fibers, the general regulatory mechanism and the ways it is disrupted in these pathologies is not yet understood. The association of DDRs with this large number of human diseases makes it an attractive cell-surface target for therapeutic interventions.
REFERENCES


