Phosphoproteomic analysis identifies insulin enhancement of discoidin domain receptor 2 phosphorylation

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**Abbreviations:** DDR2, discoidin domain receptor 2; IR, insulin receptor; LC/MS/MS, liquid chromatography tandem mass spectrometry; IMAC, immobilized metal affinity chromatography; RTK, receptor tyrosine kinase

The discoidin domain receptors (DDRs) are a unique class of receptor tyrosine kinases (RTKs) that bind to and are activated by collagen rather than soluble growth factors. Upon engagement with activated collagen rather than soluble kinases (RTKs) that bind to and are activated by collagen rather than soluble growth factors.1 Upon engagement with collagen, the receptor displays delayed and sustained tyrosine phosphorylation leading to the propagation of downstream signaling networks. DDR2 is one of two members of this class of RTKs that is commonly expressed in cells of mesenchymal origin and is activated by fibrillar collagens and collagen X.1,2 DDR2 has been shown to play a role in cell invasion and collagen remodeling through the regulation of matrix metalloproteases and collagen fibrillogenesis.3-7 While much work has been done to elucidate the extracellular collagen binding properties of DDR2, there is very limited information about the intracellular interaction partners and signaling pathways activated by DDR2.

Crosstalk between RTKs mediate a large number of processes in human health and disease.8 This process is also critical for maintaining signal robustness in response to exogenous perturbations.9 The signaling pathways downstream of DDR2 crosstalk events are poorly characterized and, in particular, the specific proteins where signal integration between RTKs occurs are largely unknown. Using HEK293 cells as a model system, a previous study has shed light on the molecular interactions between the insulin and epidermal growth factor (EGF) signaling networks and how these growth factor ligands act together to amplify mitogenic signaling.10 Vogel et al. has shown that DDR1 signals independently of the epidermal growth factor receptor (EGFR) and stimulation of cells with EGF does not induce DDR1 activation.11 In this study, we sought to determine if signal crosstalk occurs between DDR2 and the insulin receptor (IR) by performing a phosphoproteomic survey of the signaling networks activated in cells co-stimulated with collagen I and insulin. HEK293 cells have previously been shown to endogenously express 9,000 copies of the insulin receptor.12 HEK293-DDR2 cells were engineered as described in the methods and upon presentation with collagen I, showed robust receptor tyrosine phosphorylation at 1 h (Fig. 1A). These cells were serum starved for 16 h prior to stimulation with 20 μg/ml of acid-soluble collagen I and/or 150 nM of insulin for 1 h (Fig. 1B). This time-point was chosen to maximize the crosstalk between the early activation of insulin signaling (minutes) and the delayed activation kinetics of DDR2 (hours).13-15 As a control, HEK293-DDR2 cells were acid treated for 1 h. Cells were lysed and subjected to stable isotope labeling with the 8-plex iTRAQ reagent before the tyrosine-phosphorylated peptides were immunoprecipitated with pan-specific anti-phosphotyrosine antibodies (see...
Figure 1. For figure legend, see page 163.
Supplemental Methods for details). The phosphorytrosine containing peptides were subjected to further enrichment using immobilized metal affinity chromatography (IMAC) prior to liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis. In total, the profiles of 22 tyrosine phosphorylation sites across two biological replicates were generated (Table S1). Analysis of the phosphoproteomic data shows that there is good reproducibility between the two biological replicates with a Pearson correlation coefficient of 0.87 (Fig. 1C). As expected, we observed a 3-fold increase in DDR2 phosphorylation at Y740 upon collagen stimulation. This is a site located on the DDR2 activation loop and is required for full activation of the receptor. Similarly, upon stimulation with insulin, we find a 3- to 5-fold increase in two sites (Y675 and Y814) on insulin receptor substrate 2 (IRS2), a well-characterized downstream substrate of the insulin receptor. The clustering analysis reveals a cluster of phosphorylation sites that are responsive to collagen I treatment but showed an enhancement in tyrosine phosphorylation upon co-treatment with insulin. Importantly, DDR2 Y740 was phosphorylated 5-fold when co-stimulated with collagen and insulin, compared with just 3-fold upon collagen treatment alone (Fig. 1E). This result indicates that the insulin signaling pathway promotes collagen I-mediated DDR2 phosphorylation at Y740. This enhanced phosphorylation required both collagen and insulin since insulin treatment alone was unable to induce DDR2 phosphorylation. To further validate this finding, we stimulated HEK293-DDR2 cells with collagen I and/or insulin for 1 to 4 h and immunoblotted with a phospho-specific antibody directed against the activation loop phosphorylation site Y740 and the 4G10 pan-specific tyrosine phosphorylation antibody (Fig. 1F). The immunoblot confirms our proteomic results and shows increased DDR2 Y740 and total receptor tyrosine phosphorylation at 1 h with collagen I and insulin costimulation compared with collagen I stimulation alone. Interestingly, we find that this increased receptor phosphorylation is sustained from 1 to 4 h, which suggests that insulin stimulation may promote DDR2 phosphorylation in a persistent manner.

Using quantitative phosphoproteomics, we show that consistent with previous reports of EGF and DDR1,11 DDR2 receptor phosphorylation is not induced by the addition of insulin alone (Fig. 1E). However, upon co-stimulation with collagen I, insulin has the capacity to enhance DDR2 tyrosine phosphorylation in its activation loop. Insulin and collagen signaling both have functional roles in cell growth and differentiation. DDR2 knockout mice exhibit dwarfism as a result of reduced chondrocyte proliferation.18 DDR2 is also critical for osteoblastic differentiation and genetic silencing of this receptor prevents differentiation in both in vitro and ex vivo models.19 Similarly, insulin signaling promotes chondrocyte and osteoblast proliferation and differentiation.20 Understanding how these two stimuli interact to modulate cellular responses will be important for developing targeted approaches to tackling diseases such as osteoarthritis and cancer.

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Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/celladhesion/article/22572
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