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PHOSPHOPROTEOMIC ANALYSIS IDENTIFIES INSULIN ENHANCEMENT OF DISCOIDIN DOMAIN RECEPTOR 2 PHOSPHORYLATION

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Keywords

Discoidin Domain Receptor 2; Insulin; Signal Transduction; Proteomics; Mass Spectrometry

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 [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 RTK 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 signaling 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 9000 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 hour (Figure 1A). These cells were serum starved for 16 hours prior to stimulation with 20μg/ml of acid-soluble collagen I and/or 150nM of insulin for 1 hour (Figure 1B). This time-point was chosen to maximize the

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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 hour. 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 supplemental methods for details). The phosphotyrosine 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 (Supplemental 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 (Figure 1C).

To visualize the phosphotyrosine signaling networks modulated by the two stimuli, we subjected the data to hierarchical clustering analysis (Figure 1D). 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 [16]. 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 [17]. 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 to just 3-fold upon collagen treatment alone (figure 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 hours and immunoblotted with a phospho-specific antibody directed against the activation loop phosphorylation site Y740 and the 4G10 pan-specific tyrosine phosphorylation antibody (Figure 1F). The immunoblot confirms our proteomic results and shows increased DDR2 Y740 and total receptor tyrosine phosphorylation at 1 hour with collagen I and insulin costimulation compared to collagen I stimulation alone. Interestingly, we find that this increased receptor phosphorylation is sustained from 1 to 4 hours, 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 (Figure 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. Interestingly, our clustering analysis also reveals that SHIP-2, an inositol phosphatase that regulates cell migration and adhesion [21, 22], displays the same phosphorylation profile as DDR2 Y740 (Figure 1D). A recent protein interactome analysis of DDR1 binders demonstrated that SHIP-2 binds with high affinity to the receptor [23]. Similarly, SgK269 (also known as PEAK1), a cytoskeletal-associated kinase which controls cell spreading and migration clusters together with DDR2 phosphorylation [24]. Taken together, our data suggests that SHIP-2 and SgK269 may be

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candidate substrates downstream of DDR2 which facilitate its functional role in cell migration. The mechanism by which insulin enhances DDR2 phosphorylation is an outstanding question. This enhancement may be the result of direct crosstalk between the insulin and DDR2 receptor signaling networks. Alternatively, it is possible that insulin alters the binding affinity of DDR2 to collagen which ultimately increases DDR2 receptor activation. It is envisioned that a higher resolution temporal analysis of collagen and insulin co-stimulation combined with DDR2 and IR depletion experiments will provide further insights into this mechanism. Elucidation of the crosstalk mechanisms between these two pathways will be key to designing novel therapeutic strategies to overcome DDR2-driven diseases such as osteoarthritis and cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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

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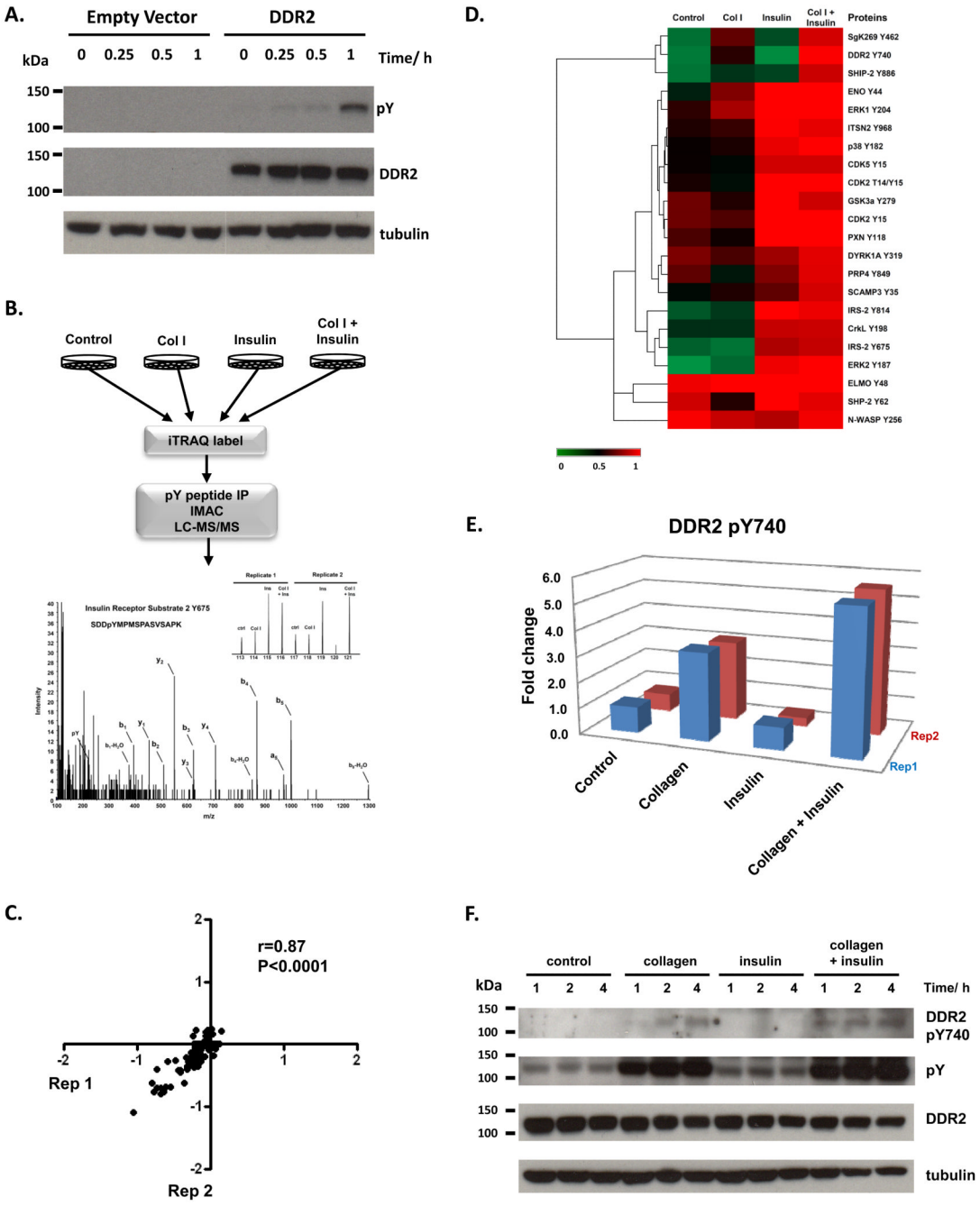

Figure 1.

(A) Immunoblot of DDR2 activation from 0-60 minutes after stimulation with 20μg/ml of collagen I. HEK293 control cells do not endogenously express DDR2. Cells engineered to express DDR2 display robust tyrosine phosphorylation upon exposure to collagen I. (B) Schematic of phosphoproteomic experimental strategy. HEK293-DDR2 cells were stimulated with acid (control), collagen I or/and insulin. Cells were then lysed, proteins digested and the resultant peptides were labeled with iTRAQ 8-plex isobaric reagents. Labeled peptides were subjected to phosphotyrosine immunoprecipitation and IMAC enrichment prior to LC/MS/MS. Phosphopeptides were sequenced and quantified as described in the methods. Mass spectrum of IRS2 Y675 highlights the peak areas for the

iTRAQ 8-plex marker ions which enable quantification of phosphorylation levels for each condition. Experiments were performed in two biological replicates. (C) Scatterplot to illustrate the reproducibility of biological replicate experiments with log10 iTRAQ ratios for the phosphoproteomic data. (D) Hierarchical clustering analysis of phosphoproteomic data comprising of 22 phosphorylation sites by Euclidean distance. Each column represents the relative phosphorylation levels in each of the four stimulation conditions (average of two biological replicates) normalized to the co-stimulation of collagen I and insulin. (E) Plot of the two biological replicates of DDR2 Y740 upon stimulation with collagen I or/and insulin. Measurements are expressed relative to the acid control. (F) Immunoblot of DDR2 phosphorylation in HEK293-DDR2 cells across 1 to 4 hours after stimulation with the indicated ligands show that phosphorylation of DDR2 Y740 and total receptor tyrosine phosphorylation levels (4G10 antibody) are increased when co-stimulated with collagen and insulin compared to collagen I alone.

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