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MTK1 signals through HER2/HER3 and heregulin to regulate extracellular acidification and cell migration

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ABSTRACT

Human MAP3K4 (MTK1) functions upstream of mitogen activated protein kinases (MAPKs). In this study we show MTK1 is required for human epidermal growth factor receptor 2/3 (HER2/HER3)-heregulin beta1 (HRG) induced cell migration in MCF-7 breast cancer cells. We demonstrate that HRG stimulation leads to association of MTK1 with activated HER3 in MCF-7 and T-47D breast cancer cells. Activated HER3 association with MTK1 is dependent on HER2 activation and is decreased by pre-treatment with the HER2 inhibitor, lapatinib. Moreover, we also identify the actin interacting region (AIR) on MTK1. Disruption of actin cytoskeletal polymerization with cytochalasin D inhibited HRG induced MTK1/HER3 association. Additionally, HRG stimulation leads to extracellular acidification that is independent of cellular proliferation. HRG induced extracellular acidification is significantly inhibited when MTK1 is knocked down in MCF-7 cells. Similarly, pre-treatment with lapatinib significantly decreased HRG induced extracellular acidification. Extracellular acidification is linked with cancer cell migration. We performed scratch assays that show HRG induced cell migration in MCF-7 cells. Knockdown of MTK1 significantly inhibited HRG induced cell migration. Furthermore, pre-treatment with lapatinib also significantly decreased cell migration. Cell migration is required for cancer cell metastasis, which is the major cause of cancer patient mortality. We identify MTK1 in the HER2/HER3-HRG mediated extracellular acidification and cell migration pathway in breast cancer cells.

1. Introduction

Mitogen activated protein kinases (MAPKs) are regulated by various extracellular stimuli resulting from a cascade of sequential phosphorylations. MAPKs, such as the extracellular signal-regulated kinases (ERKs), are phosphorylated by MEKs and MEKs are phosphorylated by MEKKs [1]. The MEKK family of MAP3Ks was cloned based on homology to the catalytic domain of the yeast MAP3K, Ste11 [1]. MEKK4 (MAP3K4) was cloned using cDNA isolated from mouse [2], while MTK1 (MAP3K4) was cloned using human cDNA [3] and the sequence homology between the two proteins is 88% amino acid identity and 92% amino acid homology. When Ssk2 was cloned from yeast [4] it became apparent that the MEKK4 and MTK1 amino acid sequences are more homologous to yeast Ssk2p than Stellp [5]. Ssk2p is regulated by osmotic stress [3]. In yeast lacking Ssk2p, MEKK4 rescues the loss of Ssk2p resulting in p38 MAPK activation indicating that MEKK4 compliments Sskp2 in yeast [3].

The heart is one of the first organs to develop and congenital malformations occur at a rate of about one in one hundred [6]. Mutation of lysine in the active site of MEKK4 produces a kinase inactive protein. Kinase inactive MEKK4 attenuates developmental epithelial to mesenchymal transformation in mouse atrioventricular canal and ventricular heart explants [7]. A knock-in mutation of kinase-inactive MEKK4 was introduced in mice and the pups die at birth from skeletal malformations and neural tube defects [8]. These findings emphasize the importance of MEKK4 kinase activity during development. In addition to kinase activity, MEKK4 protein expression is also important in development. MEKK4 is highly expressed in the developing neuroepithelium and MEKK4 knockout mice display neural tube defects resulting in exencephaly and spina bifida [9]. MEKK4 knockout mice also display a congenital malformation of the cerebral cortex and MEKK4 RNA interference impairs neuronal cell migration [10].

Human MAP3K4 catalytic activity is activated by binding of GADD45 to the amino-terminal domain of MTK1 [11]. In contrast when the amino- and carboxyl-terminal domains of MTKs associate, this interaction is auto-inhibitory, blocking kinase activity. GADD45 association with MTK1 causes dissociation of the MTK1 amino-terminal and carboxyl-terminal domains leading to dimerization, auto-phosphorylation and activation of MTK1 [12]. Human MAP3K4 (MTK1) and the mouse homolog

Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MAP3K4, mitogen-activated protein kinase kinase kinase 4; MEKK4, mitogen-activated, extracellular signal-regulated kinase kinase; MTK1, mitogen activated protein three kinase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; Ssk2, suppressor of sensor kinase; HRG, heregulin; LC–MS/MS, liquid chromatography and tandem mass spectrometry; f-actin, filamentous actin; g-actin, globular actin; cyto D, cytochalasin D; AIR, actin interacting region; pTyr, phosphotyrosines.

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(MEKK4) regulate MKK6, which is upstream of stress activated p38 MAPK [3,11,13]. In addition, stress induced activation of MEKK4 leads to activation of MEK4/7 and JNK [14].

Receptor tyrosine kinases (RTK's) and the growth factors that regulate them, such as heregulin (HRG) are often over-expressed in breast cancer cells [15–18], leading to activation of ERK1/2 activity, cell cycle progression [19] and cell migration [20,21]. The human epidermal growth factor receptors (HER) 1–4 are required for cell proliferation and differentiation during development [22,23]. HER2 is an orphan receptor with no known ligand. HER2 can form a heterodimer with EGFR, HER3 or HER4 and is often over-expressed in breast cancer [24]. HER4 expression correlates with favorable prognosis, while EGFR, HER2 and HER3 correlate with poor prognosis in breast cancer patients [25]. The growth factor, heregulin, is a ligand for HER3 and HER4, however HER3 is not kinase active and requires hetero-dimerization with either EGFR, HER2 or HER4 for activity [26–28]. Furthermore, HER2/HER3 is the preferred heterodimer for heregulin and produces strong mitogenic signaling that is linked to cancer [28–31].

HER2 over expression in estrogen positive cells is associated with tamoxifen drug resistance in breast cancer [32–34]. The drugs trastuzumab and lapatinib show high efficacy with HER2 positive patients, however drug resistance still persists [35–39]. HER3 protein expression was shown to be up-regulated with lapatinib treatment, compensating for HER2 inhibition, and HER3 phosphorylation occurred by residual HER2 expression limiting the efficacy of lapatinib treatment [40]. Therefore, HER3 over-expression and recovery of phosphorylation appears to be a compensatory mechanism in response to drug targeting of HER2. HER3 requires the catalytic activity of other members of the HER family for phosphorylation. A unique feature of HER3 is the six YXXM binding motifs that when phosphorylated function as recruitment sites for the SH2 domain of p85 of phosphoinositide kinase 3 (PI3K) leading to increased cell motility, invasion and metastasis ([41].

Cell migration requires actin polymerization and intracellular coordination of actin binding proteins, which are regulated by HER2 and downstream signaling proteins [42]. For example, heregulin stimulation of breast cancer cells enhances the conversion of globular actin (g-actin) to filamentous actin (f-actin) increasing cell migration [43,44]. Additionally, HER3 is regulated by HRG stimulation through HER2 kinase activity, which links HER3 to actin cytoskeletal reorganization and cell migration. Ssk2p is an example of an actin binding protein and is a homolog of MTK1. Ssk2p has an actin interacting region (AIR) that is required for actin cytoskeleton recovery after osmotic stress [45]. Despite the evidence for Ssk2p involvement in actin cytoskeletal reorganization, a link between MTK1 and actin has not been established in mammalian cells. Furthermore, even though HER2 and HER3 are involved in actin reorganization and cell migration, MTK1 has not been identified in this signaling process.

Cancer cells have increased glycolytic metabolism leading to acid loading and excess protons are excreted by up-regulating proton transporters [46]. Heregulin stimulation of breast cancer cells leads to extracellular acidification of media that is dependent on HER2/HER3 activity [47]. Additionally, extracellular acidification affects cell migration and invasion [48,49]. For instance, human melanoma cells treated with acidic media excrete proteases required for migration and are more invasive [50]. With regard to HER2/HER3 signaling, although many signaling proteins have been linked to these receptors it is not clear how HRG regulates proton transporters.

Proteins that function in the MTK1 pathway have not been fully characterized nor has the regulation of MTK1 kinase activity. Previously we have shown regulation of mouse MAP3K4 (MEKK4) to be through activation of the IFNγ cytokine receptor [51] and the GPCR for angiotensin II [52]. In this study we investigated whether MTK1 is also regulated by the activation of RTK's in MCF-7 and T-47D epithelial breast cancer cells. We report the recruitment of MTK1 with only activated HER3 in response to HRG in both MCF-7 and T-47D cells. MTK1 is also required for HRG induced cell migration in MCF-7 breast cancer cells through the HER2/HER3 heterodimer. Additionally, HRG induces association of MTK1 with p85 of PI3K, likely via phosphoHER3. It has been reported that HRG stimulation leads to extracellular acidification [47] an event that is linked to cancer cell migration [46,53]. We demonstrate that knockdown of MTK1 inhibits HRG-induced extracellular acidification and cell migration. Furthermore, pre-treatment of MCF-7 cells with the HER2 kinase inhibitor lapatinib inhibits association of MTK1 and HER3. MTK1 also associates with actin through the actin interacting region (AIR) and disruption of the actin cytoskeleton using cytochalasin D inhibits MTK1 and HER3 association. Together, this report establishes MTK1 as an integral signaling protein downstream of activated HER2 and HER3, required for acidification of the extracellular environment and cell migration.

2. Materials and methods

2.1. Cell culture and treatments

HEK-293, T-47D and MDA-MB-231 cells were cultured in Dulbecco's modified Eagles medium with high glucose (DMEM) pH 7.4, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin. MCF-7 cells were maintained in the same media as T-47D cells and supplemented additionally with 10 μg/ml insulin. Prior to experimental procedures, cells were cultured in DMEM supplemented only with 1% penicillin–streptomycin for 16 h. Cells were stimulated with 10 nM heregulin-β1 (HRG) EGF-Domain (Millipore Cat # 01–201) for 12 min unless otherwise indicated, EGF 3.3 nM for 12 min, 0.3 M sorbitol for 30 min or vehicle (30% glycerol in 1× phosphate buffered saline pH 7.4) for 12 min. Pre-treatment with 250 nM lapatinib was performed during serum starvation for 16 h unless otherwise indicated. Cells were treated with 1 μg/ml Cytochalasin D for 30 min prior to addition of HRG.

2.2. Western blotting and antibodies

MCF-7 or T-47D cells were lysed in lysis buffer (70 mM β-glycerol phosphate, 1 mM EGTA, 1 mM dithiothreitol, 2 mM $MgCl_2$ and 0.5% Triton X-100) with protease inhibitors: 0.5 mM phenylmethylsulfonyl fluoride, 127.4 KIU/ml aprotinin (Calbiochem Cat # 616399), 10 μM leupeptin and with 0.5 mM sodium orthovanadate. Proteins were resolved by 5%–12.5% gradient SDS-PAGE and transferred onto Protran 0.45 μm nitrocellulose blotting membrane (BioExpress Cat # F-3120-7). Membranes were blocked with 5% non-fat dry milk in 25 mM Tris–HCl, pH 7.4, 137 mM NaCl, 2.7 mM KCl and 0.15% Tween 20 (TBS-T). Immunostaining was performed in 5% non-fat dry milk in TBS-T and detected using chemiluminescence reagent (100 mM Tris pH 8.5, 250 mM luminol, 92 mM p-coumaric acid and 0.018% H_2O_2). Images were obtained using ChemiDoc™ XRS + (BIO-RAD) and quantification was performed with Image Lab Software. After the initial immunoblots were performed, the nitrocellulose membranes were stripped at 56 °C for 1 to hour using membrane stripping buffer (12.5 mM Tris pH 6.8, 2% SDS, 0.7% β-mercaptoethanol) to remove primary and secondary antibody. Membranes were re-imaged before additional immunoblots to ensure stripping was complete. Subsequent immunoblots were then performed the same way as described above. Antibodies were purchased from Cell Signaling (anti-mouse HRP-conjugated #7076S; anti-rabbit HRP-conjugated #7074S), Millipore (phosphotyrosine mouse monoclonal Clone 4G10 #05-321), Epitomics (EGFR #1902-1, HER2 #2064-1, HER3 #1186-1, HER4 #2218-1 and HER3 pY1289 #2526-1 rabbit monoclonal antibodies), Santa Cruz Biotechnology (PI3-Kinase p85α mouse monoclonal #sc-1637), Thermo Scientific (actin mouse monoclonal #MA1-744), Sigma (Anti-FLAG mouse monoclonal #F1804) and MTK1 antibodies used were developed as we previously described [52]. All commercial antibodies were used according to manufacturer recommendations.

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