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EGF Regulation of HRPAP20: A Role for Calmodulin and Protein Kinase C in Breast Cancer Cells

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1. Introduction

The breast cancer cell genome is remarkably unstable, most likely due to early dysfunction of DNA replication, repair or recombination (Roskelley and Bissel, 2002). Accumulation of genetic alterations in the cells and/or stroma lead to development of a genetically diverse cell population characterized by uncontrolled cell proliferation (Witz, 2002; Gupta et al., 2006). Therefore, identification of genes that may be responsible for pre-disposition or facilitate progression of the disease may contribute to improvement of currently available therapeutic approaches in treatment of breast cancer.

We previously reported the identification, cloning and functional characterization of HRPAP20, which encodes for protein designated Hormone Regulated Proliferation-Associated Protein 20 (accession number: NM_ 014165; Karp et al., 2004). Our observations indicated that HRPAP20 is a regulator of proliferation, survival, and invasion in hormone-responsive breast cancer cells. Moreover, highly invasive, hormone unresponsive breast cell lines, such as MDA-MB-231 and tumor specimens of invasive breast adenocarcinomas from patients exhibited constitutively elevated levels of HRPAP20 (Karp et al., 2007). Results from an independent study conducted by another group suggested that HRPAP20 is a promising marker of tamoxifen resistance in women with ER alpha-positive breast tumors (Tozlu-Kara et al., 2007). Together, these observations suggested that elevated HRPAP20 may facilitate breast cancer progression toward a more malignant phenotype.

Other studies from our group suggested that an interaction between HRPAP20 and calmodulin (CaM) may contribute to HRPAP20-mediated biological effects in tumor cells. Furthermore, a basic amino acid residue (K73) within the putative CaM-binding domain of HRPAP20 appeared to be important for CaM-binding to the protein. CaM has been shown to influence cell cycle control and proliferation in human breast cancer cells by activating CaM-kinase II (CaMK II) and MAPK-mediated signaling pathways (Cheung , 1980; Wang et al., 1983; Rodriguez-Mora et al., 2005). The recent identification of the ErbB2/HER2/Neu, ER-α, and androgen receptor (AR) as CaM-binding proteins, has opened new areas of investigation in the regulation of signaling by CaM, particularly in hormone-responsive cancer (Cifuentes et al., 2004; Li et al., 2006; Maximciuc et al., 2006).

Overexpression or constitutive activation of the epidermal growth factor receptor (EGFR) is frequently associated with the development and progression of a number of human cancers,

including breast cancer (Garcia et al., 2006). HER-2, a member of this receptor tyrosine kinase family is overexpressed in 20% -30% of aggressive breast cancers, making it an appealing target for prognosis and therapy of the disease (Meric-Bernstam et al., 2006). These and other observations have shed light on the fact that the complex role of EGF signaling in development and progression of breast cancer is incompletely understood, and suggests that gaps exist in our knowledge of its signaling mechanisms. Therefore, experiments were conducted to evaluate a potential role of HRPAP20 in EGF-mediated signaling in hormone-dependent breast cancer cells.

Protein kinase C (PKC) is a signaling intermediate that has been linked to EGF stimulation in cancer cells. The PKC family of at least 12 serine/threonine protein kinase isoforms, has been extensively studied in the regulation of intracellular signaling in response to stimuli such as growth factors and hormones (Dekker et al., 1994; Nishizuka et al., 1995; Newton 1995, and 1997; Mellor et al., 1998). The classical and novel isoforms of PKC are targets for phorbol ester compounds, such as TPA, which are a widely studied tumor promoters that mimic the actions of diacylglycerol on PKC activation (Barry et al., 2001). Altered expression and activity of several PKC isoenzymes has been observed in numerous cancers including those affecting the lung, colon, and breast (Gordge et al., 1996; McCracken et al., 2003; Gokmen-Polar et al., 2001). It has been reported that PKC-α, -δ and -ε activate several substrates that promote breast tumor cell migration and invasion (Pan et al., 2005; Tan et al., 2006; Ways et al., 1995). Therefore, it appears that PKC isoforms have distinct roles, which relate to their unique localization and/or access to substrates upon activation (Nakagawa et al., 2005). Here we show results from experiments conducted to evaluate whether HRPAP20 is a substrate for PKC. We also demonstrate whether specific PKC isoforms activated by EGF stimulation are possibly involved in HRPAP20 phosphorylation and invasion. The observations presented in this chapter support the hypothesis that HRPAP20 is an important regulator of tumor cell signaling, which may direct malignant progression in breast cancer.

2. Experimental procedures

2.1 Cell culture and treatments

The human mammary adenocarcinoma cell line MCF-7 was maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin. MCF-7 cells were serum starved for 24 hrs in DMEM supplemented with 1% ITS+ (BD Biosciences, Bedford, MA) or 0.1% BSA. Quiescent MCF-7 cells were treated with EGF (100 ng/ml) or TPA (20 nM). Treatment of MCF-7 cells with Gö6976 (1μM) or Rottlerin (10μM) was carried out 60 min prior to stimulation with EGF (100 ng/ml for 30 min). All cells were harvested by washing with ice-cold PBS and centrifugation.

2.2 HRPAP20 plasmid construction and site-directed mutagenesis

The full length HRPAP20 cDNA was ligated into the mammalian expression vector pcDNA3.1 using the restriction sites Hind III (5′ end) and Eco-RI (3′ end). The construct was sequenced and used for transfection of MCF-7 cells. For bacterial expression of recombinant HRPAP20-GST fusion protein, the restriction sites Bam-H1 and Eco-RI were engineered on the 5′ and 3′ terminals of the protein frame of HRPAP20 respectively. This oligonucleotide was generated by PCR and ligated into the bacterial expression vector PGEX-4T1 (Amersham Biosciences, Piscataway, NJ). The vectors pcDNA 3.1 and PGEX-4T1 encoding

HRPAP20 were used as templates for site-directed mutagenesis. Alanine substitutions were performed using the Quick-Change point-mutation kit (Stratagene, La Jolla, CA.). The following primers were synthesized to generate the variant HRPAP20 oligonucleotides. K73A: 5′-GAGATGTATATGTCAATTCCGCAGATCCGGTGCCTTCCT-3′. R66A: 5′-ACAAGCTGTTGTCCTTACTAGCAGATGTATATGTCAATTCC-3′. Reverse primers were synthesized complementary to the forward primers. The HRPAP20 sequence harboring both K73A and R66A site-mutations was generated by using HRPAP20/K73A as a template using primers encoding for the R66A mutation. A PCR reaction using cycling conditions recommended by the manufacturer was performed. Digestion of the parental plasmid template was accomplished using Dpn I. The site mutants were transformed into E-coli, and colonies selected on ampicillin-containing soft agar. The individual colonies were grown and the plasmid constructs isolated using Qiagen plasmid Mini-prep kit protocol. All constructs were sequenced at the University of Cincinnati DNA core facility to confirm the amino acid substitutions. The appropriate plasmids were then used for stable transfection of cells, or for bacterial expression of variant HRPAP20-GST fusion proteins.

2.3 Recombinant HRPAP20-GST protein expression and purification

Recombinant HRPAP20-GST fusion protein was produced by transformation of the Escherichia coli strain BL21-Star/p-RARE with the plasmid vector PGEX-4T1 encoding for wild-type or variant HRPAP20. The construct was sequenced at the University of Cincinnati's DNA Core Facility prior to transformation. Bacteria transformed with the empty vector alone were used to express recombinant GST alone, which was used as an assay control. The transformants were grown in complete LB broth, and IPTG (5mM) was used to induce protein expression at 27˚C. Following 4 hrs of induction, the cells were lyzed by freeze-thawing (3X) and sonication (60V, 5 cycles). Sonicates were centrifuged and GST or HRPAP20-GST fusion proteins in the supernatants were purified using glutathione-agarose (Amersham). Bound GST or HRPAP20-GST protein was eluted by reduced glutathione (20mM) and quantitated using Bradford reagent (Biorad, Hercules, CA). The purity and molecular weights of the proteins were confirmed by SDS-PAGE followed by Coomassie blue staining.

2.4 CaM-Sepharose binding analysis

The assay was performed as described previously (Boehning et. al, 2004; Karp et al., 2007). Briefly, 20 µl of CaM-Sepharose-4B beads (1.3 mg/ml, Amersham) were equilibriated with Buffer A. The equilibriated beads were then blocked for 1 h at room temperature with 1% BSA, followed by washing 1X with 500 µl of buffer A. Binding of HRPAP20-GST to CaM was evaluated by incubating 5 µg of HRPAP20-GST (wild-type or variant) or GST-only with CaM-Sepharose for 1 h at 4°C in buffer A ± $CaCl_2$ (5 mM). Bound protein was eluted using SDS sample buffer, followed by SDS-PAGE analysis. Immunoblotting with α-GST or α-HRPAP20 was used to identify HRPAP20 binding to CaM.

2.5 *In vitro* PKC kinase assay employing HRPAP20 as a substrate

The assay was performed as described in (Li and McNulty et al., 2005). Briefly, 5 µg of the fusion protein HRPAP20-GST or GST in 100 µl of PKC buffer containing 20 mM HEPES (pH: 7.4), 1.67 mM $CaCl_2$,10 mM $MgCl_2$, 1 mM DTT, 1mM ATP and 10 µCi of [γ-^{32}P] ATP was used for the kinase reaction. Myristoylated peptide PKC inhibitor (Promega, Madison, WI)

was used at a concentration of 100 mM. The kinase reaction was initiated by adding 1 µl of PKC (Promega), followed by incubation at 30 ^{o}C for 30 min. A duplicate set of the above reactions with the substitution of [γ-^{32}P] ATP with non-radioactive ATP was performed in parallel for immunoblotting analysis. The reactions were stopped by placing on ice. The fusion proteins were isolated using glutathione agarose, followed by washing, elution and SDS-PAGE. The resolved proteins were transfered to PVDF membranes and subjected to autoradiography, or immunoblotting with α-GST. The immunoblotted membranes were subsequently stripped and reprobed with α-HRPAP20.

2.6 Immune-complex kinase assay

Cells were harvested and lyzed using phosphorylation lysis buffer containing 50 mM HEPES (pH: 7.5), 150 mM NaCl, 200 µM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 1 mM EDTA, 1.5 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, and protease inhibitors (Rahman et al., 2001). Lysates were immunoprecipitated using α-PKC-A3 (Santa Cruz Biotechnology, Santa Cruz, CA) and protein-G agarose. Immune-complexes were washed with kinase wash buffer: Tris HCl (50 mM , pH 7.4), NaF (10 mM), sodium orthovanadate (1 mM), EDTA (0.5 mM), EGTA (0.5 mM), magnesium chloride (2 mM), leupeptin (10 µg/ml), PMSF (1mM) as previously described (Arya et al., 2004). 5 µg of recombinant HRPAP20-GST or GST protein, in a kinase reaction buffer containing 20 mM Tris-HCl (pH: 7.4), 10 mM $MgCl_2$ and 1 mM DTT was incubated with ATP (1 µM), γ-^{32}P-ATP (10 µCi) for 30 min at 30^{o}C. The reactions were terminated by placing on ice, followed by SDS-PAGE and autoradiography as described in previous section.

2.7 Cell transfection

MCF-7 cells were transfected with wild-type HRPAP20, R66A, K73A+R66A, or empty vector (pcDNA 3.1) alone, according to manufacturer's protocol using Lipofectamine® (Invitrogen, Carlsbad, CA). Following transfection, all cells were subjected to G418 selection (250 µg/ml) to generate stably transfected cell lines. Cells were transiently transfected with wild-type and dominant-negative PKC-δ constructs or empty vector pcDNA3.1, and used in experiments 72 hrs after transfection (previously described in Kruger et al., 2003).

2.8 Co-immunoprecipitation/immunoblotting (co-IP/IB)

Following treatments, cells were harvested using ice-cold PBS and centrifugation; then lysed in a buffer containing 10 mM Tris (pH 7.4), 0.15 M NaCl, 5 mM EDTA and 1% Triton X-100. The lysates were pre-cleared with normal rabbit serum and protein G (Upstate, Lake Placid, NY), then immunoprecipitated using α-HRPAP20 antiserum (Karp et. al, 2004) and protein G. The immunoprecipitates, together with lysates, were resolved using 12% SDS-PAGE gels, then immunoblotted with α-CaM (Upstate, Charlottesville, VA.). The membranes were subsequently stripped using a buffer containing 62.5 mM Tris (pH 6.8) and 2% SDS, then reprobed with α-HRPAP20.

2.9 Cell invasion analysis

The invasive capacity of the breast tumor cell lines was measured utilizing an in vitro transwell assay (Repesh et al., 1989, Karp et al., 2007). Briefly, 2.5 x 10^5 cells, in DMEM containing 0.1% BSA, were added to the upper well of transwell chambers (Corning Inc.,

Corning, NY) containing an 8 m porous membrane, previously coated with type I collagen, fibronectin, and Matrigel ® (Becton Dickinson Biosciences, San Diego, CA). Lower chambers contained DMEM containing 0.1% BSA and 10% FBS as a chemoattractant. Following 24 hrs, invading cells were fixed with 4% paraformaldehyde, stained with crystal violet, and counted microscopically (20X magnification) in five random fields/membrane.

3. Results

3.1 EGF regulates the interaction between HRPAP20 and CaM in MCF-7 cells

We previously showed that HRPAP20 interacts with Ca^{2+}/CaM in purified preparations and in MCF-7 cells (Karp et al., 2007). To determine whether this interaction was influenced by mitogenic stimulation such by EGF, co-immunoprecipitation/ immunoblotting (co-IP/IB) experiments were conducted using MCF-7 cells. As shown in Fig. 1, CaM co-immunoprecipitated with HRPAP20 in lysates from control cell suggesting that the two proteins existed as a complex in unstimulated cells (time 0) consistent with our previous observations. Stimulation with EGF (100 ng/ml) rapidly disrupted the HRPAP20:CaM ($p<0.05$). Thus, mitogenic stimulation appeared to cause dissociation of the protein complex that was observed in unstimulated cells. These observations suggest that HRPAP20 may function as a signaling intermediate coupled to activation of the EGFreceptor.

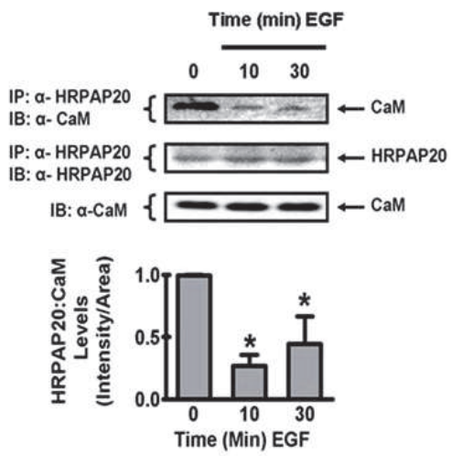

Fig. 1. EGF stimulation disrupts HRPAP20:CaM in MCF-7 cells. MCF-7 cells, previously cultured for 24 hr in serum-free medium, were treated with h-EGF (100 ng/ml). The cells were harvested at the indicated times following EGF stimulation. Cell lysates were immuno precipitated with α-CaM (upper panel). Membranes were stripped and reprobed with α-HRPAP20 (middle panel). 10% of total protein used for immunoprecipitation was resolved in parallel as an input control and Immunoblotted with α-CaM (lower panel). Densitometric analysis of three separate experiments is presented. *$p<0.05$ vs CTL.

HRPAP20:CaM interaction requires basic residues K73 and R66 in MCF-7 cells. Experiements were conducted to determine which amino acid residues within HRPAP20 were required for its interaction with CaM. Studies by others (Bagchi et al., 1992) showed that certain basic residues within the CaM-binding domains of several interacting proteins were required for their interaction. K73, R66, and V70 (hydrophobic) were identified as

potential critical residues within HRPAP20 for its interaction with CaM based upon comparison with other binding proteins (Fig. 2).

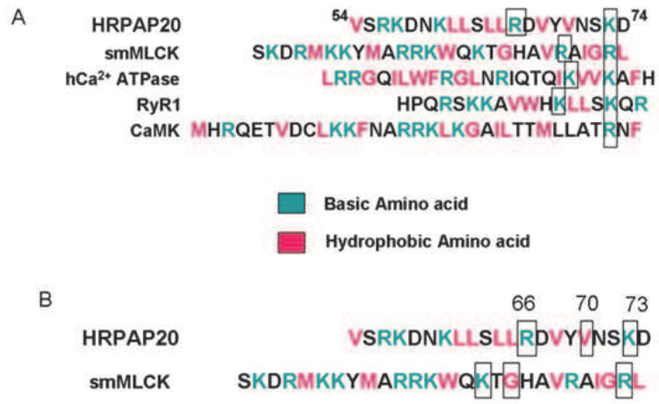

Fig. 2. Sequence of the HRPAP20 CaM-binding domain. (A) The amino acid sequence of HRPAP20 (residues 54-74) consisting of the predicted CaM-binding motif, was aligned with those of other well-characterized CaM-binding proteins. While there is no sequence conservation observed in most of the CaM-binding domain, they are characterized by a typical positioning of a C-terminal basic residue, preceded by 2-3 hydrophobic residues. (B) Sequence alignment of the CaM-binding motif of HRPAP20 with that present in smMLCK indicating positions of basic and hydrophobic residues on smMLCK that are critical for CaM-binding (Bagchi et al., 1992). Analogous residues within HRPAP20 are boxed and the positions numbered.

Previously, we showed that alanine (ala) substitution of K73 resulted in diminished CaM-binding of HRPAP20 in MCF-7 cells (Karp et al., 2007). Using site-directed mutagenesis, we generated a series of HRPAP20 variants harboring ala substitutions at these residues (HRPAP20-K73A, HRPAP20/R66A or HRPAP20/K73A+R66A, and HRPAP20/V70A). The proteins were expressed as GST-fusion proteins, then the purified recombinant proteins were evaluated by CaM-Sepharose pull-down analysis to assess their capacity to bind to CaM. The results indicated that HRPAP20-K73A exhibited ~50% reduction in CaM-binding compared to the wild-type protein (Fig. 3). Moreover, ala substitutions at K73 and R66 in combination inhibited the HRPAP20:CaM interaction to the same extent as R66A, indicating that each of these residues may contribute to CaM-binding (Fig. 3). Alanine substitution of the hydrophobic residue V70 did not significantly affect CaM-binding of HRPAP20, suggesting that this residue may not be required for in HRPAP20:CaM complex formation (results not shown).

To determine whether the HRPAP20/R66A or HRPAP20/K73A+R66A exhibited a diminished interaction with CaM in MCF-7 cells, we conducted co-IP/IB experiments using cells stably transfected with HRPAP20, R66A, K73A+R66A, or the empty vector as a control. The results demonstrated that CaM co-immunoprecipitated using α-HRPAP20, in MCF-7/HRPAP20 cells, consistent with our previous observations. In contrast, significantly reduced CaM binding was observed in cells transfected with HRPAP20/R66A or HRPAP20/K73A+R66A compared to the level observed in wildtype HRPAP20 (Fig. 4A and D). The membranes from Fig. 4A were subsequently stripped and reprobed with

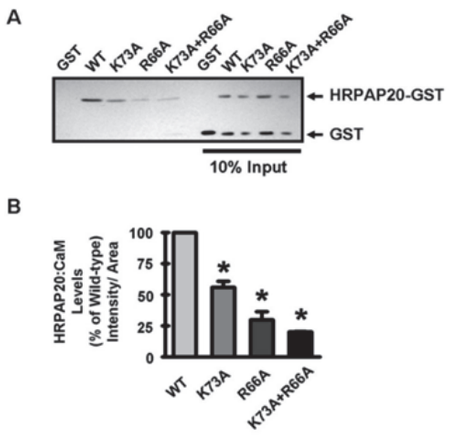

Fig. 3. K73 and R66 regulate HRPAP20:CaM interact1on in vitro. (A) 5 µg of recombinant HRPAP20-GST [wildtype-WT, or HRPAP20 harboring the substitutions K73A, R66A, or K73A+R66A] or GST alone were incubated with CaM-Sepharose in the presence of Ca^{2+} (5 mM). Eluted proteins were resolved bySDS-PAGE, then immunoblotted with α-GST. 10% of each protein used in pull-down assays was evaluated in parallel as an input control. (B) Cumulative results of densitometric analysis of three separate experiments is presented. *$p<0.05$ vs. WT.

α-HRPAP20 to confirm HRPAP20 immunoprecipitation in each of the samples (Fig. 4B). Equal concentrations of lysate protein from each of the transfectants were immunoblotted using α-CaM as an additional control (Fig. 4C). Together, these results suggested that the basic residues K73 and R66 in the CaM-binding motif of HRPAP20 may regulate its interaction with CaM in MCF-7 breast cancer cells.

Shown in Fig. 4E are results from in vitro invasion analysis of cells transfected with HRPAP20 and and its variants. Here wildtype HRPAP20 significantly ($p<0.01$) increased invasion through Matrigel® coated filters in transwell chambers. In contrast, invasion observed by the cells expressing the HRPAP20 variants R66A or K73A+R66A did not differ from empty vector-transfected controls. These observations indicated that even a partial disruption of the HRPAP20:CaM interaction by mutation of the basic residues K73 and R66 blocked HRPAP20-mediated increase in invasion. Therefore, it is suggested that K73 and R66 are likely required to maintain integrity of the HRPAP20:CaM complex, which appears to be a requisite for MCF-7 invasion.

3.2 HRPAP20 is a substrate for PKC

We previously showed that HRPAP20 is a phosphoprotein and that pharmacological inhibition of PKC reduced its phosphorylation in Nb2-11 cells (Karp et al., 2004). Experiments to evaluate whether HRPAP20 is phosphorylated by PKC were conducted utilizing recombinant HRPAP20-GST fusion protein and catalytically active rat brain PKC, in the presence of cofactors and ^{32}P-ATP. As shown in Fig. 5A, HRPAP20 was phosphorylated in the presence of PKC (lane 1), but not in its absence (lane 2). Addition of a myristoylated peptide substrate, serving as a competitive inhibitor of PKC, inhibited PKC-mediated phosphorylation of HRPAP20 (lane 3). An equal amount of recombinant GST

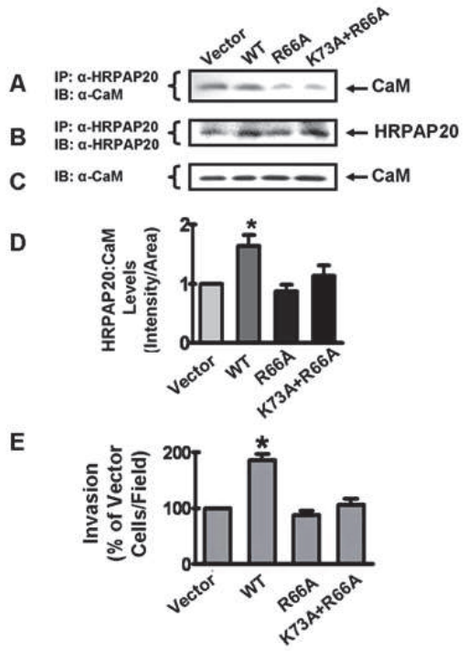

Fig. 4. Effect of HRPAP20 amino acid substitution (R66A, K73A+R66A) on HRPAP20:CaM association and invasion in MCF-7 cells. (A) MCF-7 cells, transfected with empty vector (Vector), wildtype HRPAP20 (WT), HRPAP20/R66A (R66A) or HRPAP20/K73A+R66A (K73A + R66A) were immunoprecipitated with α-HRPAP20 and immunoblotted with α-CaM. (B) Membranes were stripped and reprobed with α-HRPAP20. (C) 10% of total protein used for immunoprecipitation was resolved in parallel as an input control and immunoblotted with α-CaM. (D) Densitometric analysis of three separate experiments is presented. *$p<0.01$ WT vs Vector; $p<0.05$ WT vs K73A. (E) Cumulative results obtained from three separate in vitro invasion experiments. *$p<0.01$ WT vs Vector.

incubated with PKC represents a control reaction demonstrating the inability of PKC to phosphorylate GST (lane 4). To ensure equal loading of samples, duplicates of the above reactions with the substitution of ^{32}P-ATP with non radioactive ATP were resolved in parallel and subjected to immunoblotting with α-HRPAP20 (Fig. 5B) or α-GST (Fig. 5C). Together, these results suggest that HRPAP20 is a substrate for PKC in vitro.

3.3 HRPAP20 is phosphorylated by EGF-activated PKC

There is abundant evidence indicating an important role for EGFR and its ligands in the development and progression of human neoplasia (Johnston et al., 2006; Ji et al., 2006; Speake et al., 2005; D'Alessio et al., 2010). To evaluate whether PKC-mediated HRPAP20 phosphorylation is an event that occurrs downstream of EGF stimulation, immune-complex kinase assays were performed utilizing PKC immunoprecipitated from lysates of EGF-treated MCF-7 cells and HRPAP20-GSTas a substrate. The reactions were performed in the presence of ^{32}P-ATP, but in the absence of cofactors. As illustrated in Fig. 6A and B, HRPAP20 phosphorylation was significantly elevated upon its incubation with PKC immunoprecipitated from EGF-treated cells, compared to the level observed in control samples from untreated cells. These results suggest that PKC, activated by EGF stimulation catalyzed HRPAP20 phosphorylation.

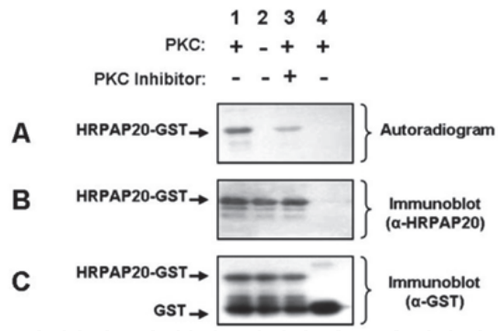

Fig. 5. Phosphorylation of HRPAP20 by PKC. (A) Recombinant HRPAP20-GST (lanes 1-3) or GST alone (lane 4) were incubated at 30°C in the presence (+) or absence (-) of purified rat brain PKC or a PKC inhibitor (myristoylated PKC peptide inhibitor, 100 μM) in the presence of [γ-^{32}P] ATP. Samples were resolved by SDS-Page followed by autoradiography. (B) Duplicates of reactions in (A), but without [γ-^{32}P] ATP, were immunoblotted with α-HRPAP20. (C) Membranes from (B) were stripped, then reprobed with α-GST.

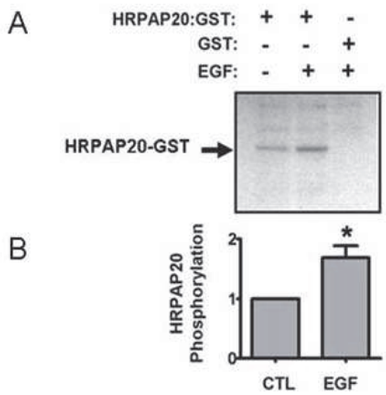

Fig. 6. HRPAP20 is phosphorylated by PKC activated subsequent to EGF stimulation in MCF-7 cells. (A) PKC from quiescent or EGF-treated MCF-7 cells was immunoprecipitated, then incubated with recombinant HRPAP20-GST or GST in the presence of γ-^{32}P-ATP, followed by SDS-PAGE and autoradiography. A representative autoradiograph indicating the level of HRPAP20 phosphorylation is shown. (B) Cumulative results from three independent experiments analyzed by densitometry are presented. *$p<0.01$ vs CTL.

3.4 PKC-δ phosphorylates HRPAP20 subsequent to EGF stimulation and is required for invasion in MCF-7 cells

The role of various PKC isoforms in the process of tumor progression in the mammary epithelium is only partially understood. However, several studies support a role for PKC-δ in breast tumor progression (McCracken et al., 2003; Nabha et al., 2005). Moreover, PKC-δ has been shown to regulate the activation of MMP-9 in breast tumor cells; an effect also observed in MCF-7 cells stably expressing elevated levels of HRPAP20 (Karp et al., 2007). Therefore, experiments were conducted to determine whether PKC-δ phosphorylated of HRPAP20 following EGF stimulation in this cell line. Quiescent MCF-7 cells were subjected to treatment with Gö6976, a pharmacological inhibitor of classic PKC isozymes, or Rottlerin, with specificity directed toward the PKC-δ isoform. The results from immunecomplex

kinase analysis indicated that while Gö6976 did not affect EGF-stimulated HRPAP20 phosphorylation, Rottlerin nearly completely blocked this effect (Fig. 7A). These results suggested that PKC-δ, activated by EGF stimulation, may be responsible for the downstream phosphorylation of HRPAP20.

To further investigate whether PKC-δ catalyzed HRPAP20 phosphorylation, MCF-7 cells were transiently transfected with constructs encoding a constitutively active wild-type (WT) PKC-δ, a kinase inactive/dominant negative PKC-δ (DN), or with the empty vector. Transfectants were treated with EGF and evaluated using immune-complex kinase analysis. The results indicated that EGF stimulated substantial HRPAP20 phosphorylation in cells expressing WT-PKC-δ (Fig. 7B). However, expression of DN- PKC-δ almost completely abolished this effect. Together, these results support the hypothesis that PKC-δ mediates phosphorylation of HRPAP20 and that this effect may underlie EGF stimulated actions.

To determine whether HRPAP20 phosphorylation affected cell invasion in vitro, invasion analysis was conducted using MCF-7 cells co-transfected with HRPAP20 and dominant negative PKC-δ. As shownin Fig. 7C, co-expression of dominant negative PKC-δ with HRPAP20, caused a significant reduction of MCF-7 cell invasion, compared to the level observed in cells transfected with HRPAP20 alone. These results suggested that activated PKC-δ is required to regulate HRPAP20, ultimately resulting in stimulating increased invasiveness in breast cancer cells.

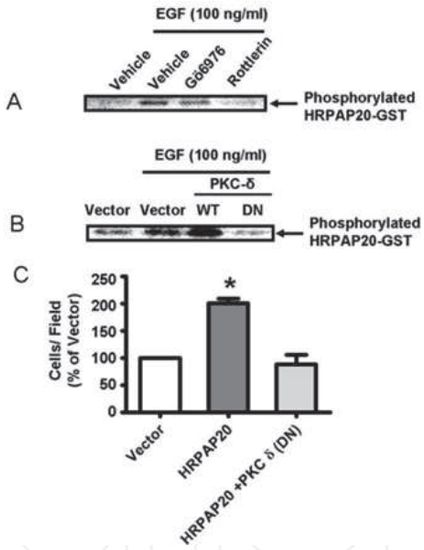

Fig. 7. PKC-δ catalyzes EGF-stimulated HRPAP20 phosphorylation and contributes to invasion. (A) Quiescent MCF-7 cells were pre-treated with PKC inhibitors Gö6976 (1 μM) or Rottlerin (10 μM), followed by the addition of EGF (100 ng/ml). PKC was immunoprecipitated and incubated with recombinant HRPAP20-GST in the presence of γ-^{32}P-ATP. A representative autoradiograph showing HRPAP20 phosphorylation is presented. (B) MCF-7 cells were transiently transfected with empty vector (Vector), wild-type (WT) or dominant negative PKC-δ (DN). Cells were then treated with EGF, followed by immunecomplex kinase analysis. (C) MCF-7 cells were transfected with empty vector (Vector), HRPAP20, or HRPAP20 and PKC-δ (DN), then evaluated by in vitro invasion assay. Cumulative results from three independently performed experiments are presented. *$p<0.01$ (HRPAP20 vs. Vector or HRPAP20 + DN-PKC-δ).

3.5 Effect of phosphorylation on HRPAP20: CaM interaction

Numerous previous studies have reported that post-translational modification may alter protein-protein interactions. Therefore, whether HRPAP20 phosphorylation affected its interaction with CaM was investigated. Experiments were conducted using MCF-7 cells treated with the phorbol ester, TPA, an activator of PKC. The HRPAP20:CaM interaction in these cells was evaluated by co-IP analysis. As demonstrated in Fig. 8A, TPA rapidly stimulated a significant ($p<0.01$) reduction in HRPAP20:CaM complex suggesting that PKC-catalyzed phosphorylation of HRPAP20 may reduce its binding capacity to CaM. Moreover, this observation correlated with the pattern of HRPAP20:CaM disruption observed upon EGF stimulation in these cells.

Furthermore, CaM Sepharose pull-down experiments were conducted to determine whether PKC-mediated phosphorylation of HRPAP20 affected its CaM-binding in vitro. The results obtained indicated that HRPAP20, phosphorylated by PKC prior to incubation with CaM, exhibited markedly reduced HRPAP20:CaM complex formation, in contrast to that observed with the native protein (Fig. 8B). This observation is consistent with our previous results suggesting that PKC likely regulates HRPAP20:CaM association. Taken together, the results suggest that HRPAP20 may modulate signaling involved in growth factor/hormone-responsive cancers by associating with PKC and CaM.

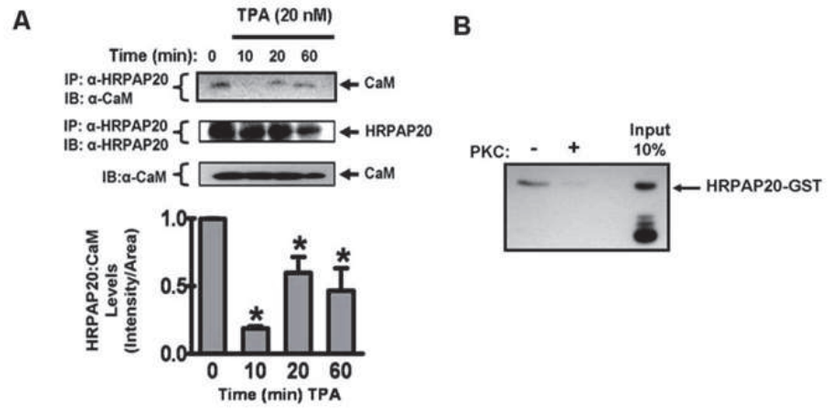

Fig. 8. HRPAP20:CaM is likely regulated by PKC-catalyzed phosphorylation. (A) Serum starved MCF-7 cells were treated with TPA (20 nM). Cells were harvested at the indicated time points, followed by immunoprecipitation of lysates using α-HRPAP20, then immunoblotted with α-CaM (upper panel). Membranes were stripped and reprobed with α-HRPAP20 (middle panel). 10% of total protein used for immunoprecipitation was resolved in parallel as an input control and immunoblotted with α-CaM (lower panel). Densitometric analysis of three separate experiments is presented. *$p<0.05$ vs. 0 min. (B) 5 µg of recombinant HRPAP20-GST was phosphorylated in vitro using rat brain PKC, then subjected to CaM- pulldown assay. A representative immunoblot using α- is shown.

4. Discussion

Tumor cell responses to extracellular stimuli includes activation of kinase pathways and alteration of protein-protein interactions, ultimately resulting in modulation of biological responses leading to progression of the disease. Since previous observations implicated a

role for HRPAP20 in breast tumor proliferation and invasion, it was important to investigate the mechanisms by which this protein participated in tumor cell signaling.

Previously we showed that HRPAP20 and CaM interacted in vitro and in MCF-7 cells (Karp et al., 2007). We therefore sought to investigate whether this interaction contributed to tumor signaling and whether it was subject to regulation by growth factor stimulation.

Recent studies have broadened the view of signal transduction to encompass a complex networks, which allow interaction between discrete signaling pathways with EGFR as a key unit for integration of multiple stimuli (Prenzel et al., 2000). CaM has been shown to bind to the EGFR, as well as modulate EGF-stimulated signaling leading to tumor progression. It was therefore important to investigate whether HRPAP20 was subject to regulation downstream of EGFR activation in tumor cells. Using co-IP/IB, we showed that HRPAP20 resides within a complex with CaM in quiescent MCF-7 cells. Stimulation with EGF caused a significant reduction in HRPAP20:CaM association. These results suggest that the HRPAP20:CaM complex is likely regulated by EGF, and may serve as a potentially important step in growth factor-stimulated cell signaling.

To investigate which specific amino acid residues within HRPAP20 participate in its interaction with CaM, we compared the sequence of the CaM-binding domain of HRPAP20 to binding domains present in other CaM interacting proteins. This assessment revealed that several basic and hydrophobic amino acids were interspersed within the HRPAP20 CaM-binding motif at positions analogous to those found to be important for other CaM-binding proteins (Bagchi et al., 1992; Fitzsimons et al., 1992). Following generation of HRPAP20 variants harboring ala substitutions at suspected key locations, experiments were conducted to evaluate HRPAP20:CaM binding of the altered proteins. Results from these suggested that the basic residues, K73 and R66, located near the C-terminus of the CaM-binding domain of HRPAP20, may be critical for its interaction with CaM.

Bioinformatic analysis of the wildtype and variant HRPAP20 amino acid sequences indicated that both K73A and R66A substitutions most likely altered the length and stability of α-helices within the protein. Notably, K73 was predicted to be responsible for a bend in the strand following the α-helix that constituted the CaM-binding motif of HRPAP20 (www.igb.uci.edu/tools/scratch.html; http://npsa-pbil.ibcp.fr/cgi-bin/secpred_hnn.pl; NNPredict Query server; and http://distill.ucd.ie/porter/). Moreover, alanine substitution of R66 reduced the number of its residue contacts, hence potentially destabilizing the helix and potentially affecting its ability to bind CaM.

To investigate whether abrogation of HRPAP20:CaM interaction by R66A or K73A+R66A substitutions affected tumor cell invasion, we compared the invasive capacity of MCF-7 cells transfected with either wildtype HRPAP20 or its variants. In contrast to the wildtype protein, HRPAP20/R66A or HRPAP20/K73A+R66A failed to increase invasion. Therefore, consistent with our previous observations, the results suggest reports an upstream requirement for CaM-binding of HRPAP20 that is coupled to tumor invasiveness.

Protein phosphorylation has been known to play a significant role in the regulation of numerous aspects of cellular function such as growth, metabolism, motility, survival, and apoptosis. Moreover, numerous protein-protein interactions have been shown to be regulated by phosphorylation (Cho et al., 2004). Since HRPAP20 appears to complex with CaM and contains three consensus motifs for PKC catalyzed phosphorylation, it was hypothesized that HRPAP20 function may be regulated by CaM-binding and phosphorylation. EGF has been reported to activate PKC in MCF-7 cells (Mueller et al., 1997). Increased levels of PKC expression and activation have been associated with

malignant transformation in a number of cell lines including breast, lung and gastric carcinomas (O'Brian et al., 1989; Takenaga et al., 1986; Schwartz et al., 1993). These observations suggest that PKC contributes to tumor progression and may serve as a therapeutic target for treatment of breast cancer.

We proposed that EGF stimulation in MCF-7 cells may subsequently induce HRPAP20 phosphorylation, following activation of PKC. To test this hypothesis, we conducted immune-complex kinase assays utilizing recombinant HRPAP20 and PKC immunoprecipitated from MCF-7 cells treated with/without EGF. Indeed, the results generated suggest that PKC immunoprecipitated from EGF-treated MCF-7 cells catalyzed HRPAP20 phosphorylation. Therefore, it appeared likely that EGF stimulation, which subsequently activates PKC, may lead to downstream phosphorylation of HRPAP20.

Results provided by others have suggested that the PKC-δ isoform is a positive regulator of metastatic progression and a survival factor in breast cancer (McCracken et al., 2003). Several reports now suggest that PKC-δ plays an important role in EGFR-mediated breast tumor progression as an important contributor to the later stages of cell migration (Kruger et al., 2003). Results presented here from immune-complex kinase assays suggested that while PKC-δ inhibition by rottlerin abrogated EGF-stimulated HRPAP20 phosphorylation, inhibition of conventional PKC isoforms with Gö6976 had no effect. This suggests that PKC-δ but not -α, -β, or –γ may mediate EGF-induced HRPAP20 phosphorylation. To further investigate HRPAP20 phosphorylation, immune-complex kinase assays were conducted using PKC immunoprecipitated from MCF-7 cells transiently transfected with wildtype or dominant negative PKC-δ. The results indicated that while expression of wildtype PKC-δ enhanced EGF-mediated HRPAP20 phosphorylation, the dominant negative PKC-δ variant failed to produce this effect. Together, the results strongly indicate that PKC-δ, activated upon EGF stimulation, may be responsible for HRPAP20 phosphorylation.

In breast cancer cell lines and fibroblasts, EGF stimulation has been shown to induce chemotactic migration with significant involvement of PKC α, ζ, δ and ε (Rabinovitz et al., 1999; Joberty et al., 2000; Iwabu et al., 2004). To determine whether PKC-δ is required for the observed HRPAP20-mediated increase in MCF-7 cell invasion, in vitro invasion experiments were conducted using cells co-expressing HRPAP20 and the dominant negative PKC-δ construct. It was observed that cells co-expressing DN PKC-δ with HRPAP20, failed to increase invasion above levels found in empty vector transfectants. These observations suggest that HRPAP20 phosphorylation by PKC-δ may be required for breast tumor cell invasion. Together, the results suggest that HRPAP20 may participate in EGF-stimulated signaling pathways in tumor cells, and that the effects of HRPAP20 may be regulated at least in part by CaM and PKC-catalyzed phosphorylation.

Phosphorylation has been shown to affect the CaM-binding affinity of several proteins. The myristoylated alanine rich C kinase substrate (MARCKS) protein and its homologue, MARCKS related protein (MRP), have been shown to bind CaM in a Ca^{2+}-dependent manner. Importantly, their interaction with CaM was diminished upon phosphorylation by PKC (Porumb et. al, 1997; Mc Ilroy et al., 1991). Several hormones produce an increase in intracellular Ca^{2+}, which facilitates CaM-binding to its target proteins and the consequent activation of PKC. These and the previous observations implicate that activation of PKC by various external stimuli may result in release of its substrates from CaM-bound pools by virtue of their phosphorylation. On the other hand, this may, in effect, result in the increase in intracellular levels of freely accessible CaM, leading to amplification of CaM-dependent processes.

Based on these observations and results indicating that HRPAP20 was a substrate for PKC downstream of EGF stimulation, we investigated whether phosphorylation affected CaM-binding of HRPAP20. Our results indicated that TPA caused a disruption of the HRPAP20:CaM complex in MCF-7 cells, in a manner similar to that observed upon EGF stimulation. In addition results from CaM-Sepharose pull-down assays illustrated that the phosphorylated form of HRPAP20 exhibited diminished CaM-binding in vitro, compared to the unphosphorylated protein.

We suggest that EGF stimulation, which activates PKC, may subsequently result in HRPAP20 phosphorylation. Furthermore, our observations clearly indicate that PKC-catalyzed phosphorylation of HRPAP20 regulates HRPAP20:CaM complex formation, and may potentially serve as an activation intermediate that leads to tumor invasion. Finally, our results suggest that a requirement of PKC-δ together with elevated HRPAP20 as factors that enhance breast cancer cell invasion. These observations provide additional clues with regard to the mechanism of regulation of HRPAP20 function and invasion in breast cancer. Thus, further studies on the role of HRPAP20 in tumor progression may reveal a hitherto unsuspected target for therapy of breast cancer.

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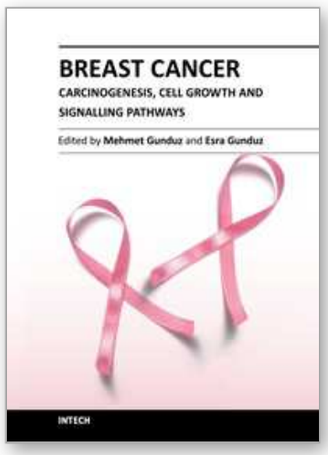

Breast Cancer - Carcinogenesis, Cell Growth and Signalling Pathways

Edited by Prof. Mehmet Gunduz

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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various aspects of breast cancer carcinogenesis from clinics to its hormone-based as well as genetic-based etiologies for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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