

## Direct Modulation of the Protein Kinase A Catalytic Subunit $\alpha$ by Growth Factor Receptor Tyrosine Kinases

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### ABSTRACT

The cyclic-AMP-dependent protein kinase A (PKA) regulates processes such as cell proliferation and migration following activation of growth factor receptor tyrosine kinases (RTKs), yet the signaling mechanisms that link PKA with growth factor receptors remain largely undefined. Here we report that RTKs can directly modulate the function of the catalytic subunit of PKA (PKA-C) through post-translational modification. In vitro kinase assays revealed that both the epidermal growth factor and platelet derived growth factor receptors (EGFR and PDGFR, respectively) tyrosine phosphorylate PKA-C. Mass spectrometry identified tyrosine 330 (Y330) as a receptor-mediated phosphorylation site and mutation of Y330 to phenylalanine (Y330F) all but abolished the RTK-mediated phosphorylation of PKA-C in vitro. Y330 resides within a conserved region at the C-terminal tail of PKA-C that allosterically regulates enzymatic activity. Therefore, the effect of phosphorylation at Y330 on the activity of PKA-C was investigated. The  $K_m$  for a peptide substrate was markedly decreased when PKA-C subunits were tyrosine phosphorylated by the receptors as compared to un-phosphorylated controls. Importantly, tyrosine-phosphorylated PKA-C subunits were detected in cells stimulated with EGF, PDGF, and Fibroblast growth factor 2 (FGF2) and in fibroblasts undergoing PDGF-mediated chemotaxis. These results demonstrate a direct, functional interaction between RTKs and PKA-C and identify tyrosine phosphorylation as a novel mechanism for regulating PKA activity. *J. Cell. Biochem.* 113: 39–48, 2012. © 2011 Wiley Periodicals, Inc.

**KEY WORDS:** PROTEIN KINASE A; PLATELET DERIVED GROWTH FACTOR RECEPTOR; EPIDERMAL GROWTH FACTOR RECEPTOR; PEPTIDE GROWTH FACTORS; SIGNAL TRANSDUCTION

Protein kinases are enzymes that transmit signals throughout the intracellular environment. They catalyze the transfer of a phosphate group from ATP to serine, threonine, and tyrosine residues within downstream substrates and in this manner alter the biochemical properties of the target proteins. Cyclic-AMP-dependent protein kinase A (PKA) is a member of the large family of AGC protein kinases that also includes PKC, PKB, Rsk, and many others. It is one of the most well studied protein kinases to date with a multitude of structural and biochemical studies providing detailed information about how the enzyme functions [Taylor et al., 2005;

Taylor et al., 2008]. PKA exists in cells as an inactive holoenzyme consisting of a dimer of two allosteric R (regulatory) subunits, each of which binds to a C (catalytic) subunit. The catalytic subunit (PKA-C) is composed of a conserved catalytic core (aa. 40–300) that is flanked by short amino and carboxy terminal sequences, termed N and C tails. It is now appreciated that the C-tail (residues 301–350) contains particular regions that function as cis-acting regulatory components of PKA catalytic activity [Kannan et al., 2007; Taylor et al., 2008]. An acidic cluster of amino acids (residues 327–336, FDDYEEEEIR), termed the active site tether (AST) is highly dynamic

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and regulates ATP binding, organization of the active site and the recognition and recruitment of peptide and protein substrates [Chestukhin et al., 1996; Batkin et al., 2000; Kannan et al., 2007; Taylor et al., 2008; Kennedy et al., 2009; Yang et al., 2009]. In cells, the catalytic subunit is assembled as a fully phosphorylated and active enzyme that is kept dormant by its association with an R subunit, in a holoenzyme complex. PKA is typically activated in response to extracellular cues that induce the production of the second messenger, cyclic adenosine monophosphate (cAMP). cAMP binds to the R subunits which induces a dramatic conformational change and the subsequent release of the active C subunits. PKA-C is then free to phosphorylate serine and threonine residues on numerous intracellular substrates that can be found in the cytosol, cytoskeleton, plasma membrane, and nucleus. The discrete subcellular compartmentalization of PKA by the A kinase anchoring proteins (AKAPs) provides an important degree of specificity to PKA signaling by helping to match a given extracellular stimulus to a specific subset of cellular targets [Scott, 2003]. PKA-mediated signal transduction controls a myriad of cellular processes including gene transcription, proliferation, differentiation, migration, and survival.

Growth factors are one type of extracellular cue known to activate PKA [Fishman et al., 1997; Ciardiello and Tortora, 1998; Bornfeldt and Krebs, 1999]. Soluble peptide growth factors such as epidermal growth factor (EGF) and platelet derived growth factor (PDGF), initiate their effects upon binding to their cognate receptor tyrosine kinases (RTKs), the EGF receptor (EGFR) and PDGF receptor (PDGFR), respectively. Once active, these receptors initiate a host of downstream signaling pathways (e.g., AKT/PKB, PKC, and Erk) which promote gene transcription, cell proliferation, survival, and migration [Hubbard and Miller, 2007]. There are numerous reports that demonstrate a role for PKA in signaling events that occur downstream of the activated EGFR and PDGFRs. A prime example is the demonstration that the RI and C subunits of PKA physically interact with the activated EGFR in a Grb2-dependent manner [Ciardiello and Tortora, 1998]. This interaction was reportedly important for the ability of the EGFR to promote mitogenic signaling events [Ciardiello et al., 1999]. PKA can directly phosphorylate the EGFR and inhibit its tyrosine kinase activity in vitro and cAMP analogs attenuate EGF-induced tyrosine phosphorylation of the EGFR in mammalian fibroblasts [Barbier et al., 1999]. However, PKA's effect on the EGFR may be cell type specific as PKA was shown to stimulate tyrosine phosphorylation of the EGFR resulting in enhanced kinase activity in PC12 and A431 cells [Piiper et al., 2003]. In response to stimulation of cells with PDGF, PKA is activated and translocated from the cell membrane [deBlaquiere et al., 1994; Graves et al., 1996], and it can either promote or inhibit cellular proliferation and migration depending upon the cell type studied [Graves et al., 1993; deBlaquiere et al., 1994; Bornfeldt et al., 1995; Graves et al., 1996; Bornfeldt and Krebs, 1999; O'Connor and Mercurio, 2001; Stork and Schmitt, 2002; Howe et al., 2005; Jalvy et al., 2007; Deming et al., 2008]. While these connections have been known for some time, the precise manner in which growth factor receptors and PKA activity intersect is poorly understood. The results reported here demonstrate that tyrosyl phosphorylation of PKA regulates its activity and identify a molecular mechanism for crosstalk between growth factor RTKs and PKA signaling networks.

## MATERIALS AND METHODS

### ANTIBODIES AND OTHER REAGENTS

Primary antibodies were obtained commercially from Santa Cruz Biotechnologies (PKA-C $\alpha$ , cat # SC904; GFP cat # SC9996), Upstate Biotechnology (phospho-tyrosine, 4G10), and Molecular Probes (GFP, cat # A1112), Cell Signaling Technologies (phospho-PKA substrate, cat # 9624L). Horseradish peroxidase-conjugated secondary antibodies were from Jackson Immunolaboratories. Platelet-derived growth factor type BB (PDGF-BB) was from Upstate Biotechnology, EGF was from Peprotech, Fibroblast growth factor 2 (FGF2) was from Sigma. Protein G beads were from Calbiochem. Recombinant untagged PKA-C $\alpha$  was obtained from New England Biolabs and purified active GST-PDGFR and GST-EGFR were from Cell Signaling Technologies. Fibronectin was from BD Biosciences. The site directed mutagenesis kit was from Stratagene. Protease arrest was from G Biosciences and sodium orthovanadate was from Sigma.

### CELL CULTURE AND TRANSFECTION

COS7 and REF52 cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum. NIH 3T3 fibroblasts were grown in DMEM plus 10% bovine calf serum. COS7 cells were transfected with FuGENE HD reagent (Roche Applied Science) following the manufacturer's instructions.

### DNA CONSTRUCTS

The plasmid encoding the PKA-C $\alpha$ -YFP was a gift from M. Zaccolo (University of Padua) and has been previously described [Zaccolo and Pozzan, 2002]. The plasmid encoding the PKA substrate GFP<sub>227</sub>RRRRSII [Yang et al., 1999] was obtained from Kevan Shokat (UCSF). The mouse (His)<sub>6</sub>-tagged PKA-C $\alpha$  construct in pET15b was a gift from Susan Taylor (UCSD). The Y330F PKA-C $\alpha$  mutant was generated via site directed mutagenesis using forward 5'-CTTTGACGACTTTGAGGAGGAAGAG-3' and reverse 5'-CTCTTCCT-CCTCAAAGTCGTCAAAG-3' primers and mouse PKA-C $\alpha$  cDNA in pET15b as a template.

### PROTEIN PURIFICATION

Recombinant GFP<sub>227</sub>RRRRSII was produced in *E. coli* strain JM109 as previously described [Yang et al., 1999]. Recombinant (His)<sub>6</sub>-tagged PKA-C subunits were produced in *E. coli* strain BL21 by inducing protein expression with 0.2 mM IPTG at 18°C overnight. (His)<sub>6</sub>-tagged proteins were purified with His-Select Beads (Sigma) following the manufacturer's protocol. The purity of the recombinant proteins was checked by SDS-PAGE and Coomassie staining.

### PHOSPHORYLATION OF PKA-C $\alpha$ BY PDGFR AND EGFR

For in vitro kinase assays with the receptors and PKA-C subunits, 200 ng of receptor was incubated with 500 ng of C subunit in kinase reaction buffer (20 mM Tris-Cl, pH 7.4, 100 mM NaCl, 200  $\mu$ M ATP, 15 mM MgCl<sub>2</sub>) at 32°C for 1 h.

**PKA Kinase Assay.** For in-vitro PKA activity assays from whole cell extracts (WCE), cells were serum starved overnight, stimulated with growth factor for the various times indicated and then washed with ice cold PBS two times. Cells were harvested in 200  $\mu$ l PKA Activity Buffer (50 mM Tris pH 7.5, 0.5 mM EDTA, 50 mM  $\beta$ -glycerolphosphate, 1 mM NaF, 0.5 mM EGTA, Protease Inhibitor Cocktail (Pierce)) and allowed to incubate on ice for 10 min. Cells were then lysed by sonication using a Virsonic 100 Ultrasonic Cell Disruptor (VirTis) by pulsing for 10 s on setting 7 twice. Protein concentration was assayed using a standard BCA protocol (Pierce). For each reaction, 100 ng of PKA substrate (GFP<sub>227</sub>RRRRSII) pre-bound to 25  $\mu$ l of His-Select HF Nickel Affinity Gel (Sigma) in PKA Activity Buffer was mixed with 10  $\mu$ g of whole cell extract in 75  $\mu$ l PKA Activity Buffer containing kinase reaction mix (15 mM MgCl<sub>2</sub>, 200  $\mu$ M ATP, and 0.5 mM DTT, final concentrations). The samples were incubated at 32°C for 20 min, after which the reaction was stopped by the addition of 5 X Laemmli sample buffer. Samples were then subjected to SDS-PAGE and immunoblot analysis using anti-phospho-PKA substrate and anti-GFP antibodies (to ensure equal loading of substrate). Densitometry was performed using ImageJ analysis software. The relative amount of growth factor-induced PKA kinase activity was calculated by normalizing to the unstimulated control. The GFP<sub>227</sub>RRRRSII substrate was highly specific for PKA in this assay as incubation with protein kinase inhibitor peptide (5–24, BioMol) completely ablated phosphorylation (Supplemental Fig. 1).

#### IN-GEL DIGESTION, MASS SPECTROMETRY, AND DATA ANALYSIS

Cubed coomassie-stained gel bands of the catalytic subunit of murine PKA alpha that had previously been subjected to an in vitro kinase reaction with or without PDGFR were rinsed with water; destained with 50% acetonitrile (MeCN), 50% ammonium bicarbonate; dehydrated with 100% MeCN; and subjected to in-gel digestion with 6 ng/ $\mu$ l sequencing grade modified trypsin (Promega), in 50 mM ammonium bicarbonate for 16 h at 37°C. Peptides were extracted once with 50% MeCN, 2.5% formic acid (FA); and once with 100% MeCN. Dried peptides were resuspended in 2.5% MeCN, 2.5% FA, and loaded using a Micro AS autosampler (Thermo Electron) and a Surveyor MS Pump Plus (Thermo Electron) onto a nano-electrospray microcapillary column packed with 12 cm of reverse phase MagicC18 material (5  $\mu$ m, 200Å, Michrom Bioresources, Inc.). Elution was performed with a 5–35% MeCN (0.15% FA) gradient over 45 min, after a 15 min isocratic loading at 2.5% MeCN, 0.15% FA. Solvent A was 2.5% MeCN, 0.15% FA and Solvent B was 99.85% MeCN, 0.15% FA. Mass spectra were acquired in a LTQ XL linear ion trap mass spectrometer (Thermo Electron). Throughout the entire run a precursor survey (MS1) scan was followed by seven data-dependent MS/MS scans on the most abundant ions (dynamic exclusion repeat count 1 and duration 180 s) as well as three MS/MS scans on the following m/z values 1,267.4, 876.4, and 1,027.4 (each  $\pm$  1.5). These values correspond to the average molecular mass of putative phosphotyrosyl-tryptic peptides harboring pY236 or pY248 (triply-charged, oxidized methionine); pY307 (doubly-charged); and pY330 (doubly-charged). Mass spectral data were searched against a murine PKA-C alpha protein database using Turbo SEQUEST (Thermo Electron, Version 27, Revision 12) requiring no enzyme

specificity, a 2 Da precursor mass tolerance, and a 1 Da fragment ion mass tolerance. Cysteine residues were required to have a static increase in 71.0 Da for acrylamide (C<sub>3</sub>H<sub>5</sub>ON) adduction. Differential modification of 16.0 Da on methionine and 80.0 Da on serine, threonine, and tyrosine was permitted.

#### GENERATION OF PHOSPHORYLATION SITE SPECIFIC pY330 PKA-C POLYCLONAL ANTIBODY

A polyclonal antibody specific for phosphorylated Y330 (pY330) on PKA-C was generated by Yenzym Antibodies (San Francisco, CA). Briefly, a peptide corresponding to phosphorylated mouse PKA-C $\alpha$  at Y330 (FDDpYEEEIR) was synthesized, conjugated to KLH, and used to immunize rabbits following a standard immunization protocol. Crude sera were collected and tested for immunoreactivity by ELISA. Sera were then pooled and affinity purified using the immunizing phospho-peptide followed by absorption with an unphosphorylated peptide matrix to remove cross-reactive antibodies.

#### KINETIC ANALYSIS OF PKA-C SUBUNITS

Kinetic analysis of control or pre-phosphorylated C-subunits were performed by radioisotope [ $\gamma$ -<sup>32</sup>P] ATP labeling using the peptide TQAKRKKSLAMA as a substrate as previously described [Dostmann et al., 1999]. The V<sub>max</sub> and K<sub>m</sub> values were derived using nonlinear regression analysis in GraphPad Prism software. A sigmoidal dose response (variable slope) was generated.

#### ASSESSMENT OF TYROSINE PHOSPHORYLATED PKA-C FROM WHOLE CELL EXTRACTS, CELL BODIES, AND PSEUDOPODS

Proteins were isolated from cell bodies (CB) and pseudopods of NIH 3T3 fibroblasts responding to a chemotactic gradient of PDGF as previously described [Howe et al., 2005]. Briefly, serum starved cells were replated for 2 h on fibronectin-coated, 3  $\mu$ m-pore polycarbonate membranes in Costar Transwell inserts. PDGF (10 ng/ml) was added to the lower chamber for 1 h. To harvest pseudopodia (Pd), inserts were washed in PBS, CB were removed from the upper surface, and the Pd on the underside were scraped into lysis buffer. Alternatively, Pd were removed, and CB on the upper surface were harvested. WCE were prepared from COS7 cells by rinsing twice with ice cold phosphate buffered saline following by harvesting in modified RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% Nonidet, 0.5% NaDOC, 5 mM EDTA, protease arrest cocktail, and 1 mM sodium orthovanadate). REF 52 cells were harvested in RIPA buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Nonidet, 0.5% NaDOC, 5 mM EDTA, protease arrest cocktail, and 1 mM sodium orthovanadate). All lysates were clarified by centrifugation and protein content quantified by bicinchoninic acid assay (Pierce). Antibodies directed against PKA-C and GFP/YFP, were used to immunoprecipitate endogenous PKA-C and the PKA-C-YFP fusion protein, respectively. Briefly, cell extracts were incubated with PKA-C or GFP antibody for 1 h at 4°C. Protein G beads were then added for an additional 30 min after which the immunoprecipitates were washed three times with lysis buffer. Immunoprecipitates and protein extracts were subjected to SDS-PAGE and immunoblot analysis using the antibodies indicated in the figure legends.

## PROTEIN DATABASE

The accession number for crystal structure of the  $\alpha$  catalytic subunit of PKA used in this study is PBD:3FJQ.

## RESULTS

### RTKS PHOSPHORYLATE PKA-C IN VITRO

Our recent work established an important role for PKA in regulating cytoskeletal events and membrane dynamics during PDGF- and EGF-mediated chemotaxis [Howe et al., 2005; Deming et al., 2008]. However, the mechanism/s by which the PDGFR and EGFR communicate to PKA was not defined. Given that the C subunit of PKA was shown previously to physically interact with the EGFR, we wondered whether the catalytic subunit might be also be phosphorylated directly by RTKs. To investigate this possibility, we performed in vitro kinase assays with purified PKA-C $\alpha$  subunits and the active cytoplasmic domains of PDGFR $\beta$  and EGFR and monitored tyrosine phosphorylation via western blot using an anti-phosphotyrosine antibody. As shown in Figure 1A, the C subunit of PKA (molecular weight 38 kD) was tyrosine phosphorylated by both the PDGFR $\beta$  and EGFR. There was no phosphotyrosine signal at 38 kD in the samples containing RTK alone. The  $\alpha$  PDGFR like the PDGFR $\beta$ , also phosphorylated PKA-C albeit to a lesser extent (Fig. 1B). Taken together, these results demonstrate that both the EGFR and PDGFR are able to phosphorylate PKA-C in vitro.

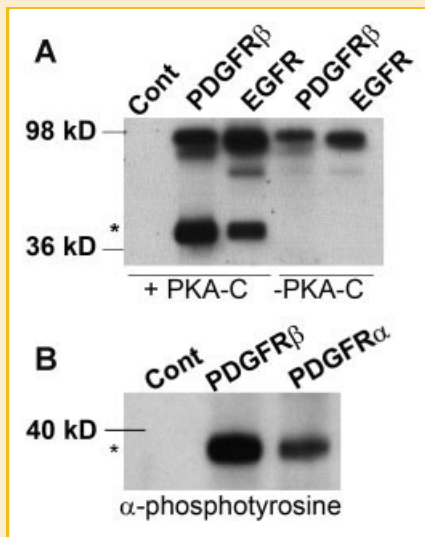


Fig. 1. A: Buffer (Cont), active GST-tagged-PDGFR $\beta$  (PDGFR $\beta$ ) or EGFR (EGFR) were subjected to a cold kinase assay in the presence (+PKA-C) or absence (-PKA-C) of purified PKA-C $\alpha$ . The samples were then processed for immunoblot analysis using an antibody that recognizes pan phosphotyrosine. Asterisk (\*) denotes the position of PKA-C (mw 38 kD). The upper band migrating near the 98 kD marker corresponds to the molecular weights of the active, tyrosine-phosphorylated, bacterially purified, GST-receptors. B: Recombinant PKA-C $\alpha$  was incubated with buffer only (Cont) or bacterially purified, GST-tagged PDGFR $\beta$  or PDGFR  $\alpha$  under cold kinase assay conditions and samples were processed as described in (A).

### PKA-C SUBUNIT IS TYROSINE PHOSPHORYLATED IN GROWTH FACTOR STIMULATED CELLS

In order to determine whether this tyrosine phosphorylation event on PKA-C occurs in cells undergoing growth factor stimulation, the phosphorylation status of PKA-C following treatment of cells with EGF was investigated. Exogenously expressed YFP-PKA-C was immunoprecipitated from unstimulated and EGF-stimulated COS7 cells, known to express the EGFR, and then assessed for total phosphotyrosine via western blot using a pan anti-phosphotyrosine antibody. While there was no detectable tyrosine phosphorylation on YFP-PKA-C in unstimulated cells, tyrosine phosphorylation was slightly detectable at 10 min and increased by 20 min following stimulation with EGF (Fig. 2A). The previously demonstrated role for PKA in growth factor-induced chemotaxis [Howe et al., 2005] and

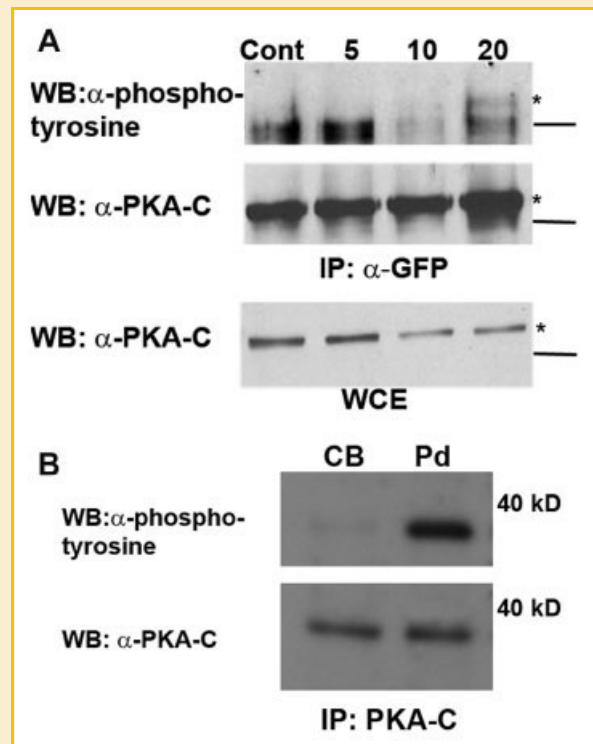


Fig. 2. A: COS7 cells were transfected with a plasmid encoding PKA-C $\alpha$  fused to YFP. Twenty four hours later cells were serum starved overnight and then treated with vehicle control (Cont) or EGF (100 ng/ml) for 5, 10, or 20 min after which they were harvested in mRIPA buffer. The PKA-C $\alpha$ -YFP fusion protein was immunoprecipitated, then subjected to SDS-PAGE and immunoblot analysis using antibodies directed against pan-phosphotyrosine (upper panel) and the catalytic subunit of PKA-C $\alpha$  (middle panel). The lower panel illustrates the amount of PKA-C $\alpha$ -YFP in the whole cell extract and is equivalent to 1/20th the input. The lines to the right represent the position of a 60 kD molecular weight marker and the asterisk (\*) denotes the PKA-C $\alpha$ -YFP fusion protein. The experiment was repeated three times and yielded similar results. B: Cell bodies (CB) and pseudopods (Pd) were isolated from NIH3T3 cells as described in Materials and Methods section. PKA-C $\alpha$  was immunoprecipitated from each of these fractions, separated by SDS-PAGE, and immunoblotted with antibodies against pan-phosphotyrosine and the C subunit (PKA-C). The numbers to the right indicate the position of a 40 kD molecular weight marker. The result shown is representative of two separate experiments.



PDGF-induced membrane ruffling in fibroblasts [Deming et al., 2008], led us next to investigate whether PKA-C may be tyrosine phosphorylated in cells undergoing PDGF-mediated chemotaxis. To address this possibility, endogenous PKA-C subunits were isolated from cell body and pseudopod extracts prepared from NIH 3T3 fibroblasts migrating toward a gradient of PDGF. Although the amount of total PKA-C was similar in the cell body and pseudopod fractions, tyrosine-phosphorylated PKA-C was relegated to the pseudopod (Fig. 2B). This together with our previous finding that PKA activity (but not total PKA-C protein levels) is markedly enriched within pseudopods suggest that phosphorylation of PKA-C may regulate PKA function during cellular migration. These results provide evidence that a pool of PKA-C becomes phosphorylated on a tyrosine residue/s in cells stimulated with EGF and PDGF.

### RTKS PHOSPHORYLATE PKA-C ON TYROSINE 330

We next wished to map which tyrosine/s on PKA-C were phosphorylated by the PDGFR and EGFR. To do this, we first subjected PKA-C to an *in vitro* kinase reaction with or without the PDGFR. The reaction was terminated by adding SDS sample buffer and heating to 95°C for 5 min. Following SDS-PAGE, PKA-C was cut from the gel and subjected to *in gel* tryptic digestion. Tryptic peptides were then subjected to liquid chromatography tandem mass spectrometry (LC-MS/MS) in a linear ion trap mass spectrometer as described in the Methods section. In addition to randomly choosing peptides for fragmentation analysis we also performed targeted MS/MS analysis on masses corresponding to phosphotyrosyl-tryptic peptides that would contain tyrosines on the surface of the enzyme based on its crystal structure. We identified one phosphotyrosyl peptide in the PDGFR-treated sample and none in mock-treated sample. The upper panel of Figure 3A shows the MS/MS spectrum of the identified phosphotyrosyl peptide harboring phosphorylated tyrosine 330. The lower panel is an MS/MS spectrum of the same peptide in its unphosphorylated state which was identified in both samples. It is clear that both phosphorylated and unphosphorylated peptides show strikingly similar collision-induced dissociation fragmentation patterns. Similar results were obtained when PKA-C was phosphorylated by the EGFR (not shown). Importantly, when Y330 was mutated to a non-phosphorylatable phenylalanine residue (Y330F) the ability of the PDGFR $\beta$  and EGFR to tyrosine phosphorylate PKA-C $\alpha$  *in vitro* was essentially ablated (Fig. 3B). These data suggest that Y330 is the major (if not only) tyrosine phosphorylated by PDGFR $\beta$  and EGFR *in vitro*. We next generated a peptide antibody targeted against phosphorylated Y330 (pY330). The antibody specifically recognized wild type PKA-C $\alpha$  subunits phosphorylated by PDGFR and EGFR *in vitro* but not control subunits or the Y330F mutant (Fig. 3C,D). The antibody was raised against a region in PKA-C that is conserved across all three isoforms (Materials and Methods section and Fig. 4A) and therefore is predicted to react with phosphorylated PKA-C $\alpha$ ,  $\beta$ , and  $\gamma$  subunits.

### TYROSINE PHOSPHORYLATION OF PKA-C ENHANCES ITS KINETIC ACTIVITY

Y330 lies within a region of the C terminal tail of PKA-C that is highly evolutionarily conserved and Y330 is found in the  $\alpha$ ,  $\beta$ , and  $\gamma$  isoforms of PKA-C (Fig. 4A). The C tail region, wherein Y330 resides

is malleable and in the closed (i.e., ATP- and substrate-bound) conformation of the enzyme, lies in close proximity to the ATP binding pocket, glycine rich loop, and p-3 subsite of the substrate (Fig. 4B). Therefore, we reasoned that phosphorylation at this residue might have effects on the activity of the enzyme. To address this possibility, the kinetic activity of unphosphorylated C subunit was compared to that of C-subunit that had been pre-phosphorylated with the EGFR. As demonstrated in Figure 4C, tyrosine phosphorylation of PKA-C altered the  $K_m$  for a known peptide substrate. Specifically, phosphorylation on PKA-C reduced the  $K_m$  by threefold, resulting in a significant overall increase in the catalytic efficiency of the enzyme. The  $V_{max}/K_m$  was 0.980 for control and 3.982 for pY-PKA-C subunits, respectively (Table I). Similar results were obtained when PKA-C was pre-phosphorylated with PDGFR $\beta$  (data not shown). Although tyrosine phosphorylation enhanced PKA kinase activity, the ability of the protein kinase inhibitor (PKI) peptide (residues 5–24 of full length PKI) to inhibit catalytic activity was not altered (data not shown). Thus, it appears that phosphorylation of PKA-C at Y330 by RTKs enhances the overall activity of the enzyme *in vitro*, in particular by increasing the affinity for substrate binding.

### GROWTH FACTORS INDUCE PHOSPHORYLATION OF Y330 IN CELLS

We next used the pY330 antibody to monitor the phosphorylation status of PKA-C upon stimulation of cells with growth factors. Consistent with the observed increase in total phosphotyrosine on PKA described in Figure 2A, stimulation of COS7 cells with EGF induced the phosphorylation of Y330 at 15 min, which remained high for at least 30 min (Fig. 5A). Assessment of EGF-induced PKA activity in COS7 cells revealed increases in PKA activity at time points coincident with the phosphorylation of Y330 (Fig. 5B). In REF 52 fibroblasts, which are known to express the PDGFR, little to no basal phosphorylation of Y330 was observed in unstimulated cells or those treated with PDGF for 10 min (Fig. 5C). However, phosphorylation of Y330 was evident 20 and 30 min following stimulation with PDGF (Fig. 5C). Interestingly, like for EGF, peak PKA activity following PDGF treatment was coincident with the timing of phosphorylation on Y330 (Fig. 5C,D). It should be noted that treatment of REF 52 cells with PDGF appeared to slightly increase the total amount of PKA-C in the whole cell extract used for immunoprecipitation (Fig. 5C), which could have contributed to the rise in activity. We also wished to determine whether phosphorylation of PKA-C on Y330 might occur in response to growth factors other than PDGF and EGF. In addition to the PDGFR, fibroblasts express the fibroblast growth factor receptor tyrosine kinase (FGFR). In NIH 3T3 fibroblasts, FGFR is activated by FGF2 and FGF2 activates PKA in these cells [Pursiheimo et al., 2000]. Therefore, the ability of FGF-2 to induce phosphorylation of Y330 on PKA-C was investigated in NIH 3T3 cells. Indeed, while there was little phosphorylation of Y330 in unstimulated NIH 3T3 cells, FGF2 increased the levels of phosphorylation at 10 and 20 min following treatment (Fig. 5E). Taken together, our results demonstrate that PKA-C subunits are phosphorylated on Y330 in cells following stimulation with three different growth factors and suggest that phosphorylation of PKA-C on Y330 may be a general intermediate event of RTK signaling.

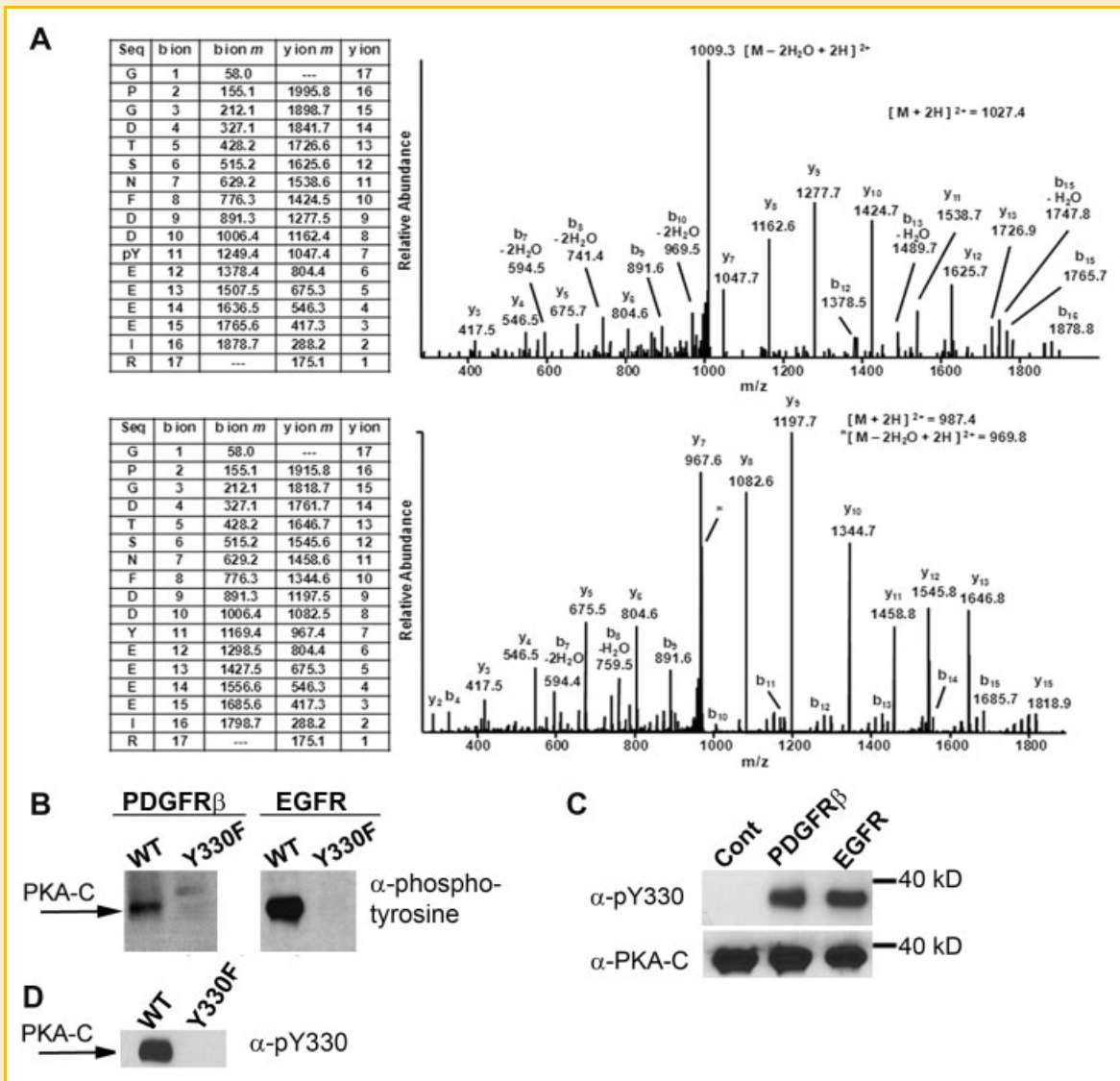


Fig. 3. A: Low energy MS/MS spectra identifying tryptic peptides with Y330 in a phosphorylated state (upper panel) or unphosphorylated state (lower panel). Sequest Xcorr scores were 3.5 and 5.7, respectively. See Methods section for details. B: Mutation of Y330 to Y330F ablates PDGFR- and EGFR-induced tyrosine phosphorylation. His-tagged wildtype or Y330F PKA-C $\alpha$  subunits were incubated together with GST-PDGFR $\beta$  or GST-EGFR and subjected to a cold in vitro kinase assay. Samples were then analyzed by SDS-PAGE and immunoblot analysis using an antibody directed against pan-phosphotyrosine. C: Phospho-specific antibody that recognizes Y330. In vitro kinase assays were performed with buffer alone (Cont), PDGFR, or EGFR and recombinant PKA-C. Immunoblot analysis was performed using the Y330 phosphospecific antibody as described in Materials and Methods section. Membranes were stripped and re-probed with an antibody that recognizes total PKA-C. D: In vitro kinase assays were performed with or EGFR and His<sub>(6)</sub>WT or His<sub>(6)</sub> Y330F PKA-C $\alpha$  subunits. Immunoblot analysis was performed using a Y330 phosphospecific antibody (pY330) as described in Materials and Methods section.

## DISCUSSION

The results presented in this report describe an unprecedented role for tyrosine phosphorylation of the C subunit of PKA during growth factor signaling and build upon the existing role of the C tail as a critical regulatory region. The C tail allosterically regulates the enzyme on two levels; it directly regulates the catalytic core and functions to facilitate interactions with cellular components that modulate catalytic activity [Kannan et al., 2007]. Our data show that RTKs directly phosphorylate PKA-C on a tyrosine residue/s and

identified Y330 within the C tail as the major phosphorylation site in vitro. We demonstrate that phosphorylation of PKA-C by RTKs functionally modifies the enzymatic activity of PKA in vitro and lastly establish that this phosphorylation event occurs in response to stimulation of cells with several different growth factors.

Although the functional relevance of the AST of PKA-C has been extensively studied with regard to how it allosterically regulates the catalytic core, post-translational modification of this regulatory region has never been reported. In mammalian cells, post-translational modification of PKA-C is reported to occur through

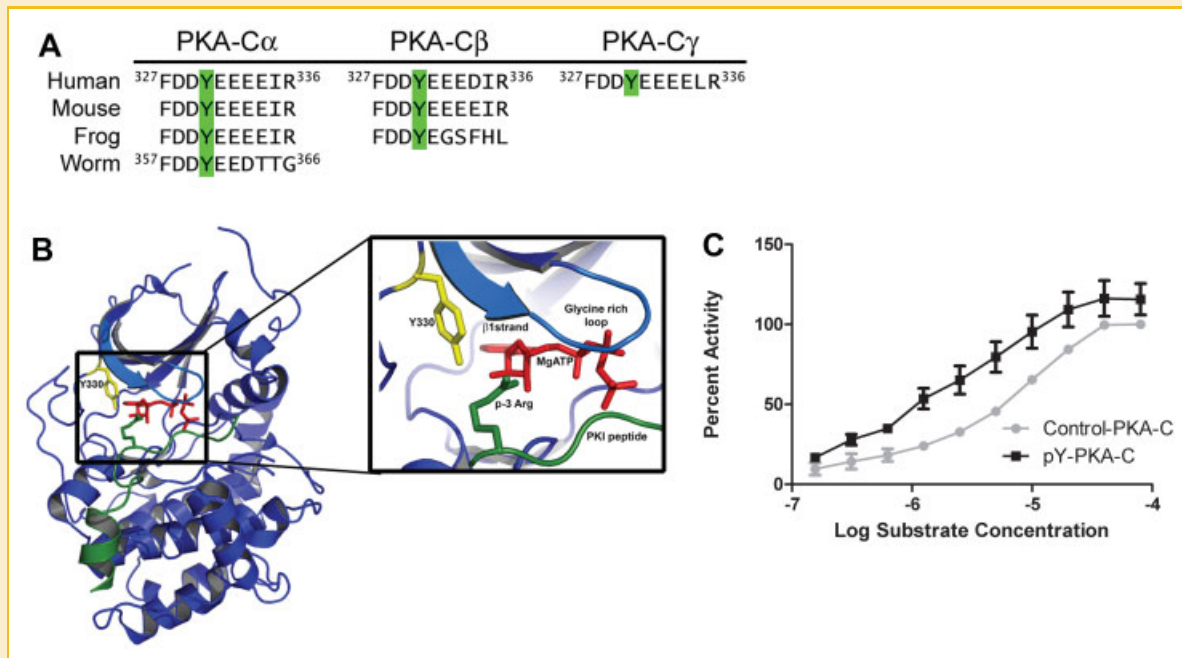


Fig. 4. A: PKA is evolutionarily conserved. The amino acid sequence in the conserved AST region of PKA-C is shown Human, Mouse, Frog, and Worm. The following NCBI sequence identifiers were used: For PKA-C $\alpha$ : Human: NP\_002721, Mouse: NP\_032880, Frog: AAI69356, Worm: CAB41352; for PKA-C $\beta$ : Human: NP\_002722, Mouse: NP\_035230.1, Frog: NP\_001080696, and for PKA-C $\gamma$ : Human: AAH39888. B: Structure of the active catalytic subunit of PKA- $\alpha$ , PDB: 3FJQ. Tyrosine 330 is highlighted in yellow. Note the close proximity of Y330 to the  $\beta$ 1 strand and the glycine rich loop (marine), and the Arginine that lies in the p-3 position of the PKI peptide inhibitor (green). MgATP is colored in red. C: Kinetic analysis of PKA-C subunits. Control or tyrosine-phosphorylated (pY-PKA-C) PKA-C $\alpha$  subunits were subjected to in vitro kinase assays as described in Methods section. The percent activity was calculated as the reaction velocity ( $\mu\text{M}/\text{min}/\mu\text{g}$ ) normalized to the maximum velocity of the control and then plotted as a function of the log of the substrate concentration ( $\mu\text{M}$ ). The graph represents the average of four independent experiments and error bars indicate the standard error  $\pm$  S.E.

autophosphorylation on serine residues (S10, S110, and S338) and phosphorylation in the activation loop on T197 by PDK1 or a PDK1-like enzyme [Yonemoto et al., 1993; Cheng et al., 1998]. Large scale phosphoproteomic screens performed in mouse brain and cell lines expressing oncogenic tyrosine kinases have also observed phosphorylation on tyrosine 69, however, the kinase/s responsible for this phosphorylation event and the functional relevance have not yet been determined [Rikova et al., 2007; Ballif et al., 2008; Guo et al., 2008]. It is formally possible that other tyrosine residues in addition to Y330 are phosphorylated by RTKs at a lower abundance. However, given that there was no detectable tyrosine phosphorylation on sites other than Y330 via mass spectrometry and that mutation of Y330 to Y330F ablated phosphorylation, it is likely that Y330 is the major, if not only, residue phosphorylated by the PDGFR and EGFR.

The kinetic studies described here demonstrate that tyrosine phosphorylation of the catalytic subunit enhances the enzymatic activity of PKA. Y330 specifically appears to be involved in the hydrophobic packing of the backbone of the  $\beta$ 1 strand that flanks the glycine rich loop (Fig. 4B) [Yang et al., 2009]. During the catalytic cycle, Y330 moves into close proximity to the p-3 subsite of the substrate, a site known to confer specificity for PKA [Kemp et al., 1977; Taylor et al., 1990; Walsh et al., 1992]. A previous report by Batkin et al. demonstrated that mutation of Y330 to a panel of amino acids resulted a marked increase in the  $K_m$  and decrease in catalytic efficacy of PKA-C [Batkin et al., 2000]. Interestingly, of the

mutants tested, the phenylalanine mutant (Y330F) exhibited the smallest although yet significant difference in  $K_m$  as compared to control PKA-C. As conversion of tyrosine to phenylalanine retains the phenyl ring but loses the phenolic hydroxyl these results suggested that both the phenyl ring and phenolic hydroxyl of Y330 contribute to the  $K_m$  of substrate. This is thought to occur through packing or Van der Waals interactions between the phenyl ring and residues in the conserved core and hydrogen bonding between the phenolic hydroxyl and the basic amino acid at the p-3 position, respectively. The decreased  $K_m$  observed with the tyrosine phosphorylated PKA-C subunit are consistent with this idea that Y330 is involved in creating preferential affinity of kinase for substrate as the addition of a phosphate to the hydroxyl group would be predicted to strengthen a hydrogen bonding interaction with the basic amino acid at p-3 (Fig. 4B). The timing of Y330 phosphorylation in EGF and PDGF-treated cells was coincident with growth factor-induced PKA activity (Fig. 5A,B,C and D), and the tyrosine phosphorylated C subunit was restricted to the pseudopod of chemotaxing fibroblasts, a structure previously reported to have highly localized PKA activity [Howe et al., 2005]. However, whether phosphorylation of Y330 confers specificity for PKA to a particular subset of substrates or is required for growth factor-induced PKA kinase activity remains to be determined.

A bioinformatics study revealed that the C-tail of AGC kinases is highly conserved, suggesting a global mode of regulation for this

TABLE I. Effect of Phosphorylation at Y330 of PKA-C on  $K_m$  and  $V_{max}$  Values

	$K_m$ ( $\mu$ M)	$V_{max}$ ( $\mu$ M/min/mg)	$V_{max}/K_m$
Control PKA-C	$7.97 \pm 0.19$	$7.8 \pm 1.6$	$0.980 \pm 0.218$
pY-PKA-C	$^{**}2.61 \pm 0.59$	$9.1 \pm 2.0$	$^{*}3.982 \pm 1.343$

Nonlinear regression analysis was performed on the data represented in Figure 4C to obtain the average  $K_m$  and  $V_{max}$  for control or phosphorylated (pY-PKA-C) PKA-C $\alpha$  subunits.

Each value represents the average result ( $\pm$  S.E.) of four independent experiments. A paired Student's *t*-test demonstrated a significant difference in the  $K_m$ .

\* $P < 0.05$  between the control and pY-PKA-C subunits.

\*\* $P < 0.005$  and the  $V_{max}/K_m$ .

family of kinases [Kannan et al., 2007]. In addition to directly contributing to the catalytic efficacy of the enzyme, the C tail of some AGC kinases contains protein interaction motifs that recruit cellular regulatory factors. For example, the PXXP motif in PKC $\beta$ II was shown to bind to two chaperone proteins (Hsp90 and cdc37) and contribute to protein stability [Gould et al., 2009]. Moreover, a recent study identified a conserved FD(X)<sub>1-2</sub>Y/D motif within the tail that is essential for docking of the activating kinase, PDK1 [Romano et al., 2009]. Interestingly, in PKA, this motif encompasses Y330. In addition to modulating catalytic activity itself, it is intriguing to speculate that phosphorylation of Y330 may also recruit the binding of a protein/s (e.g., SH2 and/or PTB domain-containing proteins) to

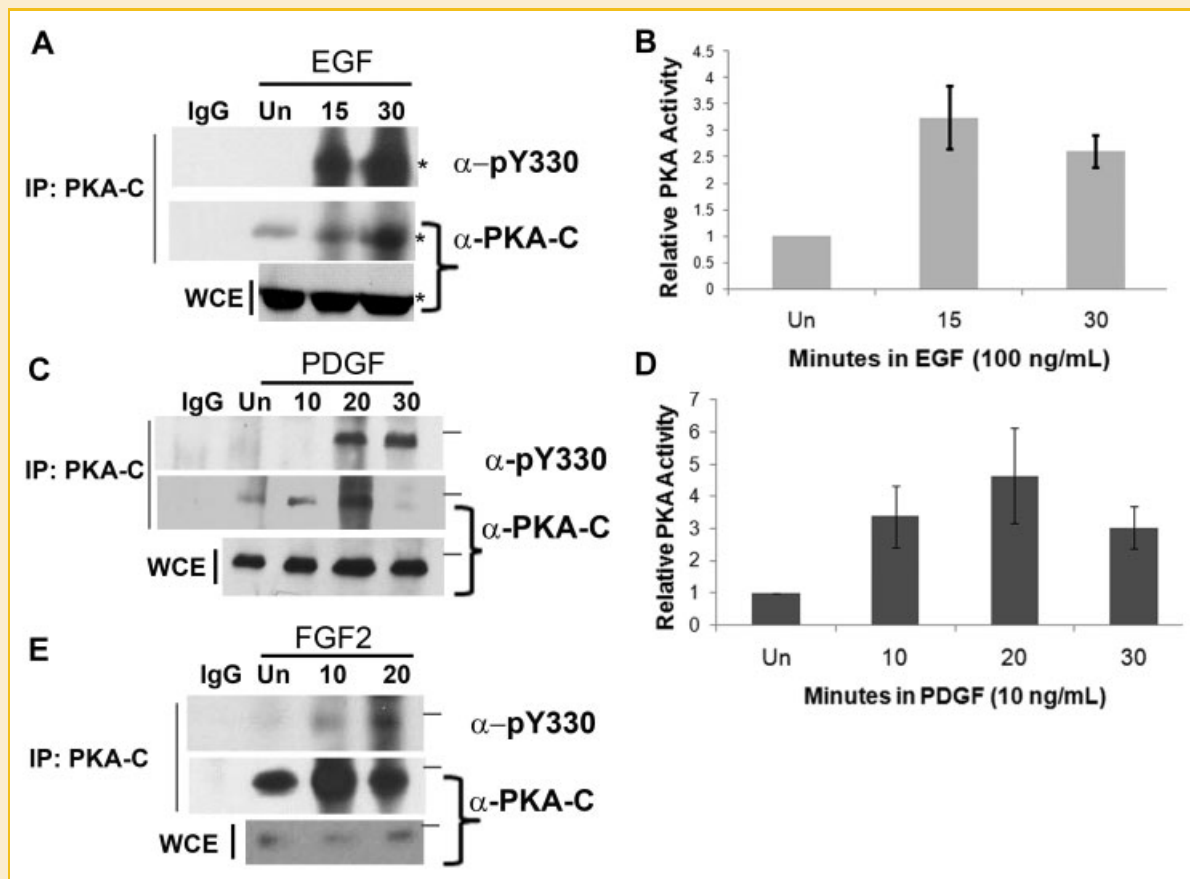


Fig. 5. A COS7 cells were transfected with a plasmid encoding PKA-C $\alpha$  fused to YFP. Twenty four hours later cells were serum starved overnight and then treated with vehicle control (Un) or EGF (100 ng/ml) for 15 or 30 min after which they were harvested in RIPA buffer. The PKA-C $\alpha$ -YFP fusion protein was immunoprecipitated, then subjected to SDS-PAGE and immunoblot analysis using antibodies directed against pY330 (upper panel) and the catalytic subunit of PKA-C $\alpha$  (middle panel). The lower panel illustrates the amount of PKA-C $\alpha$ -YFP in the whole cell extract. The results depicted are representative of three independent experiments. The asterisk (\*) denotes the PKA-C $\alpha$ -YFP fusion protein. B: COS7 cells were serum starved overnight and then treated with vehicle control (Un) or 100 ng/ml EGF for 15 or 30 min. Cells were harvested in PKA assay buffer and processed for PKA kinase activity as described in Materials and Methods section. The graph depicts the relative amount of PKA activity induced upon stimulation with EGF (100 ng/ml) normalized to the unstimulated control. The data represent the average of three independent experiments and the error bars indicate the standard error ( $\pm$  S.E.). C: REF 52 fibroblasts were serum starved overnight and then treated with vehicle control (Un) or PDGF (20 ng/ml) for the times indicated and endogenous pY330 subunits were immunoprecipitated as described in Materials and Methods section. Immunoprecipitates were subjected to SDS-PAGE and immunoblot analysis using the pY330 antibody. The membrane was stripped and re-blotted with an antibody directed against PKA-C. The lower panel depicts the amount of total PKA-C in the WCE. The line denotes the position of the 40 kD molecular weight marker. The results are representative of four independent experiments (D) REF 52 fibroblasts were serum starved overnight and then treated with vehicle control (Un) or PDGF (10 ng/ml) for the times indicated. PKA kinase activity was assessed from WCE as described in Materials and Methods section. The graph depicts the relative amount of PKA activity induced upon stimulation with PDGF normalized to the unstimulated control. The data shown represent the average of three independent experiments and the error bars indicate the standard error ( $\pm$  S.E.). E: NIH 3T3 cells were serum starved overnight and then treated with vehicle control (Un) or FGF2 (25 ng/ml) for the times indicated. Endogenous PKA-C immunoprecipitates and whole cell extracts (WCE) were subjected to SDS-PAGE and western blot analysis as described in (C). The line denotes the position of the 40 kD molecular weight marker. The results shown are representative of two independent experiments.



the C tail which would then add an entirely new element to the regulation of PKA activity. While additional experimentation will be required to determine the contributions of this phosphorylation to the spectrum of cellular events mediated by growth factors, the current results provide the mechanistic basis for crosstalk between growth factor RTKs and PKA, two major signal transduction pathways.

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