

## INSIGHT INTO THE ASSOCIATION BETWEEN PHOSPHOLIPASE C- $\gamma$ 1 AND THE INSULIN RECEPTOR

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### RESUM

La identificació de senyals intermediaris que interaccionen directament amb el receptor d'insulina (IR) és actualment objecte d'una intensa recerca, ja que sembla estar relacionada amb l'acció de la insulina. Recentment, el nostre grup de recerca va establir l'associació induïble de la fosfolipasa C (PLC)- $\gamma$ 1 amb l'IR i la seva implicació en la transducció de senyal mitjançada per la insulina a través de l'activació de la ruta de la cinasa regulada per Ras. En aquest estudi hem generat diversos mutants de la PCL $\gamma$ 1 per identificar i definir la funció dels residus tiol PCL $\gamma$ 1 en el control de la interacció IR-PCL $\gamma$ 1 i determinar l'impacte que el redox cellular té en el procés. Per aconseguir aquests objectius, hem utilitzat dos mètodes que demostren la presència de sulfhidrils en la PCL $\gamma$ 1 sensibles a l'oxidació en cèl·lules CHO que expressen IR. Diversos experiments de mutagènesi dirigida varen demostrar la modificació selectiva de les cisteïnes 8 i 12 de PCL $\gamma$ 1 després de l'estimulació amb peròxid d'hidrogen i la inhibició d'oxidases de membrana productores de ROS. Aquestes dades suggereixen que la regió aminoterminal de la PCL $\gamma$ 1 conté cisteïnes nucleofíliques particularment sensibles a la regulació redox i que són necessàries per a la interacció eficient amb l'IR. La importància d'aquestes troballes s'ha confirmat també en estudis sobre el paper regulador de la redox cellular en la transducció de senyal de la insulina.

**Paraules clau:** receptor d'insulina, fosfolipasa C, interaccions proteïna-proteïna, redox, transducció de senyal.

### SUMMARY

An intense investigation is underway to identify the signaling intermediates that interact directly with the insulin receptor (IR) as they appear to be associated with the transmission of insulin action. We recently reported the inducible association of phospholipase C (PLC) $\gamma$ 1 with

the IR and its involvement in the transduction of insulin-mediated signals through activation of the Ras/extracellular-regulated kinase pathway. Akin to protein phosphorylation, redox regulation represents an important metabolic modulator of cellular functions. In this study, various mutant forms of PCL $\gamma$ 1 were generated to identify putative key redox-sensitive cysteines that provide sites for a number of post-translational modifications. Toward this aim, two methods were used that demonstrate the presence of oxidation-sensitive cysteine residues in PCL $\gamma$ 1. Using site-directed mutagenesis experiments we found that two cysteines in PCL $\gamma$ 1 (Cys-8 and Cys-12) were modified with hydrogen peroxide and protected against a host of reactive oxygen species through pharmacological inhibition of cellular membrane oxidases. The data suggest that the amino-terminal region of PCL $\gamma$ 1 contains reactive cysteine-SH groups that are exquisitely sensitive to redox regulation and which are associated with efficient interaction with the IR. The importance of these findings is already asserting themselves in studies reporting the regulatory role of cellular redox in insulin signal transduction.

**Keywords:** insulin receptor, phospholipase C $\gamma$ 1, protein-protein interaction, oxidative stress, signal transduction.

## INTRODUCTION

The physiological effects of insulin are mediated by activation of the intrinsic tyrosine kinase function of the insulin receptor (IR) and its association with a number of scaffold molecules (e.g., IRS-1, Gab-1) harboring distinct recognition domains for the binding of signaling-competent effector proteins (Saltiel and Pessin, 2002). The insulin receptor is a heterotetrameric glycoprotein consisting of two  $\alpha$ - $\beta$  dimers linked by disulfide bonds (Garant *et al.*, 1999). We used a novel procedure to study the interaction between IR and endogenous molecules in cells by taking advantage of the fact that the homobifunctional crosslinking reagent 1,6-bismaleimido-hexane (BMH) selectively reacts with nucleophilic cysteine residues to form an irreversible link between two interacting proteins (Garant *et al.*, 2000). Immunoprecipitation-based technique coupled with mass spectrometry analysis allowed us to establish that insulin promoted the formation of a covalent complex between the IR  $\beta$ -subunit and phospholipase (PLC) $\gamma$ 1 upon addition of BMH to chinese hamster ovary (CHO) cells stably expressing the human IR (Kwon *et al.*, 2003) (see figure 1A, B). Western blot analysis independently

confirmed the recruitment of PCL $\gamma$ 1 to the IR following insulin stimulation of a number of cell types. Of significance, the amino-terminal region of PCL $\gamma$ 1, which consists of a pleckstrin homology (PH) domain and a series of EF-hands, has been proposed to contribute to the interaction between PCL $\gamma$ 1 and the activated IR (Kwon *et al.*, 2003; Bernier *et al.*, 2004).

PCL $\gamma$ 1 plays an important role in the intracellular transduction of receptor and nonreceptor tyrosine kinases. It is widely distributed and exerts essential function in mammalian growth and development as evidenced by the fact that mice deficient in PCL $\gamma$ 1 are embryonic lethal (Ji *et al.* 1997). PCL $\gamma$ 1 is a member of the family of phosphoinositide-specific PLCs that convert phosphatidylinositol 4,5-bisphosphate (PI4,5P<sub>2</sub>) to two second messengers, inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and 1,2-diacylglycerol (DAG), upon cell activation (Rhee, 2001). IP<sub>3</sub> mobilizes Ca<sup>2+</sup> from intracellular stores, while DAG is responsible for the activation of a subset of protein kinase C isoforms (e.g.,  $\alpha$ ,  $\beta$ ,  $\tau$ ,  $\epsilon$ ). Activation of a chimeric receptor containing the juxtamembrane and tyrosine kinase domains of the IR led to PCL $\gamma$ 1 activation and concomitant increase in calcium mobi-

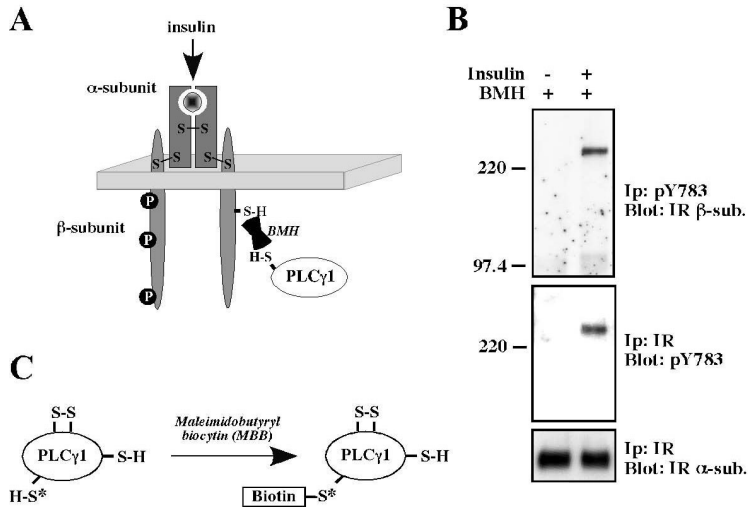


FIGURE 1. A) Schematic representation of the covalent recruitment of PCL $\gamma$ 1 to the intracellular domain of the IR  $\beta$ -subunit in insulin-stimulated cells in the presence of BMH, a homobifunctional thiol-specific crosslinking agent. B) Immunoblot analysis in support of this process. pY783, mAb antibody specific for tyrosine phosphorylated PCL $\gamma$ 1. C) Identification of putative PCL $\gamma$ 1 nucleophilic cysteines using the thiol-modifying agent MBB. \* denotes accessible cysteine(s) with low pKa at physiological pH. Panels A and B were reproduced from Bernier *et al.* (2004).

lization (Telting *et al.*, 1999). PCL $\gamma$ 1-mediated PI4,5P $_2$  hydrolysis in anti-IR immunoprecipitates has been found in insulin-stimulated 3T3-L1 adipocytes (Eichhorn *et al.*, 2001). PCL $\gamma$ 1 participates in insulin-stimulated glucose uptake in adipocytes through activation of DAG-sensitive PKC $\zeta$  (Lorenzo *et al.*, 2002), whereas microinjection of neutralizing antibodies to PCL $\gamma$ 1 interferes with insulin's ability to induce DNA synthesis in IR-expressing Rat-1 fibroblasts (Eichhorn *et al.*, 2002). Finally, knockdown of PCL $\gamma$ 1 expression by RNA interference significantly reduces activation of extracellular-regulated kinase (ERK) pathway, but not that of Akt, in response to insulin, whereas reconstitution of PCL $\gamma$ 1 in PCL $\gamma$ 1 $^{-/-}$  mouse embryonic fibroblasts elicits a marked increase in insulin-stimulated ERK activation (Kwon *et al.*, 2003). These and other data provide clear indication that increased PCL $\gamma$ 1 activity could modulate the metabolic and mitogenic effects of insulin.

The juxtamembrane region of the human IR cytoplasmic domain contains a cysteine residue that exhibits low pKa at physiological pH (Bernier *et al.*, 1995; Bernier *et al.*, 2004), which makes it a chemical hot spot for a number of biochemical interactions. Indeed, the selective sensitivity of Cys-981 to thiol-modifying agents, such as maleimidobutyryl biocytin (MBB), was used to our advantage in tagging human IR in intact cells (Garant *et al.*, 2000). However, no studies have been performed to test the presence of reactive cysteine residue(s) in PCL $\gamma$ 1, which could account for its ability to covalently bind the IR upon BMH treatment. On the basis of this, we set out experiments to identify oxidation-sensitive cysteine-SH groups in PCL $\gamma$ 1 and assess their role in the control of IR-PCL $\gamma$ 1 covalent interaction in the presence of BMH. While the mechanism of IR-PCL $\gamma$ 1 interaction remains to be fully elucidated, analysis of the cysteine residues in PCL $\gamma$ 1 that are potentially

FIGURE 2. (*facing page*) PCL $\gamma$ 1 contains reactive thiols. A) Schematic representation of various HA-tagged PCL $\gamma$ 1 constructs generated in this study. B) Thiol-specific biotinylation of wild-type and mutant forms of PCL $\gamma$ 1 in CHO-IR cells. Serum-starved cells expressing wild-type (WT),  $\Delta$ 216C,  $\Delta$ 304N, or C8A/C12A double point mutant form of PCL $\gamma$ 1 were semipermeabilized with digitonin in the presence of 100  $\mu$ M MBB for 10 min at 6  $^{\circ}$ C. Anti-HA immunoprecipitates from cell lysates were resolved by SDS-PAGE and blotted with HRP-conjugated streptavidin (SA-HRP) to detect thiol-biotinylation signals. The membrane was then reprobbed with HA antibody (lower panel). C) Serum-starved CHO-IR cells expressing empty vector (-) or HA304N (+) were subjected to MBB-induced thiol biotinylation as indicated above. Anti-PCL $\gamma$ 1 immunoprecipitates were prepared and analyzed by Western blot using SA-HRP and anti-HA antibody. D-E) CHO-IR cells ectopically expressing empty vector (pcDNA), the wild-type HA304N or various Cys->Ala mutant forms of HA-304A (C8C12AA, C106A, C247A) were incubated in the absence (-) or the presence (+) of 100  $\mu$ M BMH for 10 min at 6  $^{\circ}$ C before quenching the reaction with L-cysteine. Anti-HA immunoprecipitates (D, lower panel) or total cell lysates were analyzed by Western blot with the indicated primary antibodies.

susceptible to redox regulation may provide new insight into PCL $\gamma$ 1 function.

## METHODS

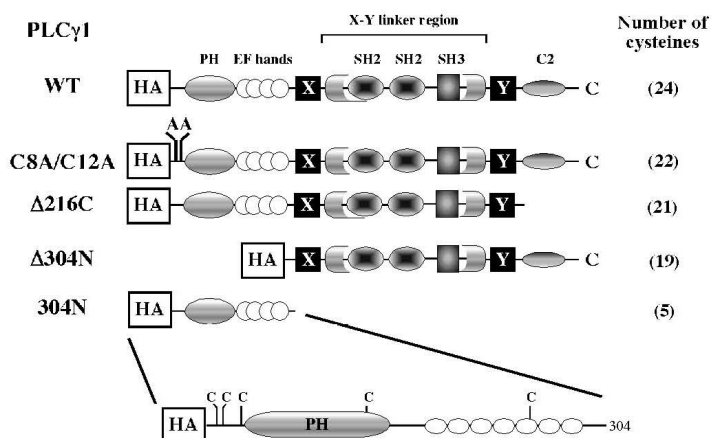
**Plasmid constructs.** C- and N-terminal fragments and a double point-mutation (C8A/C12A) of the rat PCL $\gamma$ 1 (NM\_013187) were generated using plasmid pRK5/HA-PCL $\gamma$ 1 (kindly provided by Graham Carpenter Nashville, TN, USA). The generation of HA (hemagglutinin) epitope-tagged 304N (from a.a. 1-304 of PCL $\gamma$ 1, which contains PH domain and EF-hands) involved a PCR-based site-directed mutagenesis approach to introduce HindIII restriction site between EF-hands and catalytic domain "X" of PCL $\gamma$ 1. An HindIII/HindIII fragment (2961 bp long) was excised. To generate PCL $\gamma$ 1  $\Delta$ 216C mutant (deletion of a.a. 1075-1291, which contains C2 domain), HindIII restriction site was introduced by mutating the region (3309AAGCCTTTG3317) to AAGCTTTTG. An HindIII/HindIII fragment (660 bp long) was excised. The construction of PCL $\gamma$ 1 $\Delta$ 304N mutant

was carried out as followed: Two EcoRI restriction sites were introduced by mutating the region (100TCGTCGG108) and (900ACCGGCTTC908) to GGAATTCGG and ACCGAATTC, respectively, while deleting EcoRI site present in pRK5 vector's multicloning site. An EcoRI/EcoRI fragment encompassing 304N was excised. The linearized pRK5/HA-tagged plasmids were then self-ligated. The cysteine 8 and 12 to alanine mutation in PCL $\gamma$ 1 (C8A/C12A) was introduced by replacing codon TGC with GCC. Similarly, several point-mutations of HA-304N (termed C8C12AA, C26A, C106A, and C247A) were constructed using the QuickChange site-directed mutagenesis kit (Stratagene). All of the mutations were verified by DNA sequence analysis.

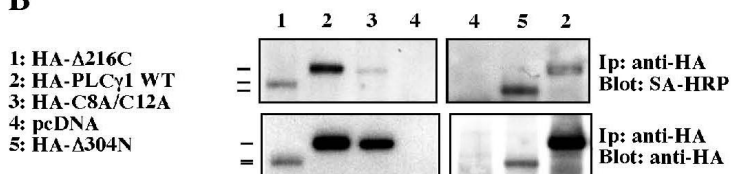
**Cell culture.** CHO cells expressing the human IR (CHO-IR) cells were maintained and grown to near confluence in Ham's F-12 medium supplemented with 10% FBS, glutamine and penicillin/streptomycin. Cells were then maintained in medium without serum (SFM) for 4h, after which 100 nM insulin was added for 5 min. In some instances, cells were treated for 30 min with either vehicle (dimethylsulfoxide, DMSO), diphenylethylidonium chloride (DPI; Alexis Corp., San Diego, CA), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; Fisher), pyrrolidinedithiocarbamate (PDTTC; Calbiochem, La Jolla, CA), bpV(phen) (Calbiochem) or thioctic acid (oxidized form) (Sigma-Aldrich, St-Louis, MO) prior to the addition of insulin.

**Transient transfection assays.** CHO-IR cells were transfected by the Lipofectamine2000 method (Invitrogen). Empty expression vector and expression plasmids encoding wild-type or mutant forms of PCL $\gamma$ 1 were mixed with the transfection reagent and directly added into the culture plates at a ratio of 2-4  $\mu$ g of each plasmid/60-mm dish. Twenty hours later, cells were serum-starved for 3h and then subjected to treatments as described below.

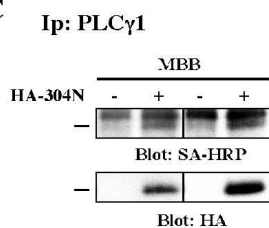
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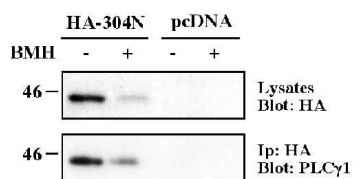
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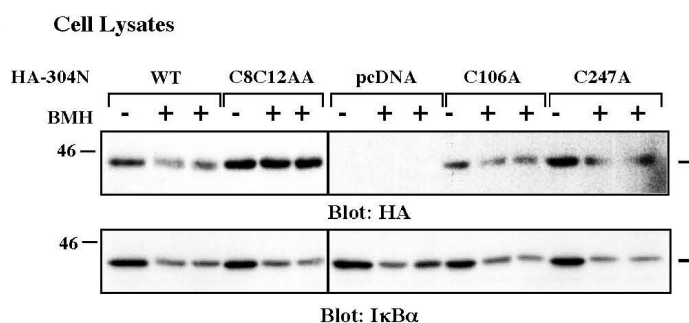
**C**



**D**



**E**



*Thiol-specific biotinylation and BMH-mediated chemical crosslinking of PCL $\gamma$ 1 in cells.* Serum-starved cells were washed twice in phosphate-buffered saline (PBS), and then incubated in Krebs Ringer Phosphate (KRP) buffer for 5-30 min at 37 °C before their transfer to thermoregulated aluminium cooling plates set at 6 °C. The thiol-biotinylation reaction was initiated by the simultaneous addition of 20  $\mu$ g/ml digitonin and 100  $\mu$ M MBB (Calbiochem) for 10 min, with subsequent addition of 4 mM L-cysteine to quench the reaction. Detection of biotinylated protein sulfhydryls was carried out by Western blot using HRP-conjugated streptavidin (Vector Lab, Burlingame, CA). The crosslinking reaction followed the procedure of Garant *et al.*, (2000). After a 5-min stimulation with insulin (100 nM) in KRP buffer, cells were transferred to 6 °C and then the crosslinking reaction was initiated by the addition of 100  $\mu$ M BMH (Pierce Chemical Corp., Rockford, IL) or vehicle (DMSO) and quenched 10 min later with 4 mM L-cysteine.

*Immunoprecipitation and Western blot analysis.* Cell lysis was performed using established procedures (Kwon *et al.*, 2003). Following appropriate treatment of the cells, cells were washed once with PBS and then lysed in radioimmune precipitation buffer containing 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 0.02% NaN<sub>3</sub>, 100 mM NaF, 1 mM orthovanadate, and protease inhibitor cocktail (Calbiochem). The clarified lysates were isolated from homogenates by centrifugation at 4 °C. An aliquot was suspended in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5%  $\beta$ -mercaptoethanol and heated at 70 °C for 10 min. A second aliquot of the clarified lysates was incubated with mAb anti-PCL $\gamma$ 1 (Chemicon International; N-terminal epitope) or mAb anti-IR (clone 29B4; Calbiochem) for 16 h at 4 °C. The samples were subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions,

transferred onto PVDF membrane and then blots were incubated with primary antibodies in blocking buffer (mAb anti-HA antibody (Covance); rabbit anti-IR  $\alpha$ -subunit and anti-I $\kappa$ B $\alpha$  (Santa Cruz Biotechnology); rabbit anti-phosphoPCL $\gamma$ 1 (pY783) antibody (BioSource International); antiphosphotyrosine (clone RC20)-linked to HRP and rabbit anti-IR  $\beta$ -subunit (Transduction Lab)). Bound antibody was detected using enhanced chemiluminescence according to established protocols.

*Assay of intracellular H<sub>2</sub>O<sub>2</sub>.* Serum-starved CHO-IR cells were incubated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> or 10  $\mu$ M DPI for 30 min before the addition of 100 nM insulin. Five min later, 4  $\mu$ M dichlorohydrofluorescein (CM-H<sub>2</sub>DCF-DA; Molecular Probes, Eugene, OR) was added in the dark for 10 min at room temperature. Fluorescence of the indicator dye was visualized using a Zeiss LSM-410 inverted confocal microscope at an excitation wavelength of 488 nm and emission at 515/540 nm.

## RESULTS

*PCL $\gamma$ 1 contains reactive thiols.* MBB is a biotin-containing, thiol-specific reagent that reacts readily with proteins containing cysteine residues. When combined with streptavidin-conjugated HRP, this approach allows the detection of redox-sensitive cysteines. MBB was used successfully to identify Cys981 of the human IR as a nucleophilic thiol (Bernier *et al.*, 1995).

Following transient expression of wild-type and mutant forms of HA-tagged PCL $\gamma$ 1 in CHO-IR cells (see figure 2A), MBB-mediated thiol-biotinylation reaction was carried out and anti-HA immunoprecipitates were evaluated for the content of reactive cysteines by Western blotting (see figure 2B). A significant incorporation of biotin was observed in wild-type PCL $\gamma$ 1 after administration of MBB to semi-permeabilized cells, which sup-

ports the notion that PCL $\gamma$ 1 contains reactive cysteine residues at physiological pH. Similar to wild-type PCL $\gamma$ 1,  $\Delta$ 304N and  $\Delta$ 216C PCL $\gamma$ 1 mutants showed strong reactivity to MBB, whereas the level of thiol-biotinylated C8A/C12A PCL $\gamma$ 1 mutant was markedly lower (see figure 2B, upper panel). Reprobing the membrane with anti-HA showed the relative expression level of each PCL $\gamma$ 1 construct (see figure 2B, lower panel). To further establish the presence of reactive cysteines in the N-terminal region of PCL $\gamma$ 1, HA-304N (which contains the PH-EF domain) was expressed in CHO-IR cells and subjected to thiol-biotinylation (see figure 2C). The results showed the conjugation of HA-304N with biotin. Moreover, the construct exhibited BMH-induced chemical crosslinking, as evidenced by the reduced signal at 43 kDa in HA immunoprecipitates (see figure 2D). As shown in see figure 2A, the N-terminal region of PCL $\gamma$ 1 contains 5 cysteines, some of which may be subject to oxidative modification. Various Cys to Ala point-mutants of HA-304N were generated and analyzed for their response to BMH-induced crosslinking in intact CHO-IR cells (see figure 2E, upper panel). The results clearly showed a sharp reduction in the recovery of monomeric forms of WT, C106A and C247A mutants following BMH treatment, while having no detectable effect on C8C12AA. C26A expression was very low and thus could not provide reliable information (data not shown). Reprobing the membrane with anti-I $\kappa$ B $\alpha$  antibody indicated that BMH was effective at promoting thiol modification of I $\kappa$ B $\alpha$  protein in each cell line (see figure 2E, lower panel). Taken together, our data revealed that PCL $\gamma$ 1 possesses reactive cysteine residues at position 8 and 12.

*Modulation of PCL $\gamma$ 1 thiol reactivity in intact cells.* Several enzymes that regulate downstream components in the insulin signaling cascade are potential targets of redox modifications that take place in cells exposed to a host of reactive oxygen and nitrogen species

(Finkel, 2003). To measure the generation of intracellular H<sub>2</sub>O<sub>2</sub>, cells are loaded with the redox indicator dye based on dichlorohydrofluorescein (CM-H<sub>2</sub>DCF-DA) that is trapped intracellularly after cleavage by cellular esterases. Following stimulation of CHO-IR cells with 100 nM insulin, an oxidant signal was detected by DCF fluorescence, which peaked at 5 min (see figure 3A, panel b), and began to dissipate by 10 min (not shown). Others have reported that the oxidant generated by insulin was H<sub>2</sub>O<sub>2</sub>, as preincubation of the cells with catalase attenuated the fluorescent signal (Mahadev *et al.*, 2001). Exogenous addition of H<sub>2</sub>O<sub>2</sub> markedly increased DCF signal (see figure 3A, panel c), whereas incubation of the cells with diphenyleneiodonium (DPI), an inhibitor of cellular NADPH oxidase activity, blocked the production of H<sub>2</sub>O<sub>2</sub> in response to insulin (see figure 3A, panel d). We then investigated the effect of DPI and H<sub>2</sub>O<sub>2</sub> on PCL $\gamma$ 1 thiol reactivity in CHO-IR cells. When cells were treated with DPI, the incorporation of MBB in wild-type PCL $\gamma$ 1 increased 3-fold while having no detectable effect on the C8A/C12A mutant (see figure 3B). Addition of exogenous H<sub>2</sub>O<sub>2</sub> caused a 50% and full reduction in thiol biotinylation of the wild-type PCL $\gamma$ 1 and C8A/C12A mutant, respectively. The data suggest that PCL $\gamma$ 1 contains a discrete subset of cysteines that are likely to be involved in the response of PLC $\gamma$ 1 to redox modification. The absence of cysteines 8 and 12 in C8A/C12A mutant confers refractiveness to DPI action.

Insulin facilitates the recruitment of PCL $\gamma$ 1 to the activated IR, enabling both proteins to be covalently linked using BMH, a selective homobifunctional thiol-specific crosslinker (Kwon *et al.*, 2003). To test whether alteration in cellular redox could affect this process, CHO-IR cells were preincubated with DPI or H<sub>2</sub>O<sub>2</sub> followed by the addition of insulin and subsequent treatment with BMH (see figure 3C). A dose-dependent enhancement in IR-PCL $\gamma$ 1 covalent association was

achieved with DPI with concomitant reduction in unconjugated IR  $\beta$ -subunit (see figure 3C, left panel). Conversely,  $H_2O_2$ , which increases cellular thiol oxidation (see figure 3B), dose-dependently reduced covalent binding of PCL $\gamma$ 1 to the IR (see figure 3C, right panel) while promoting the recovery of tyrosine phosphorylated IR  $\beta$ -subunit and PCL $\gamma$ 1 (see figure 3D).

BpV(phen) is a stable peroxovanadium compound with insulinomimetic properties by virtue of its ability to activate the IR kinase and subsequent stimulation of downstream signaling (Bevan *et al.*, 1995). Previous studies indicated that bpV(phen) induces the production of intracellular superoxide anion through activation of NADPH oxidase at the plasma membrane (Yamaguchi *et al.*, 1995), which leads to transient cytosolic acidification (Bianchini *et al.*, 1994). We next ascertain the effect of bpV(phen) on BMH-dependent IR-PCL $\gamma$ 1 crosslinking. When stimulated with insulin and challenged with 20  $\mu$ M bpV(phen), CHO-IR cells exhibited a marked attenuation in IR-PCL $\gamma$ 1 complex formation (see figure 3E, lane 2 *vs.* 1). Because antioxidants can offer cellular defense against oxidative stress (Roy *et al.*, 1997), we tested the effect of three structurally unrelated antioxidants on the cellular response to bpV(phen). While thiocetic acid ( $\alpha$ -lipoic acid) and the metal chelator PDTC were mostly ineffective at counteracting bpV(phen) signaling, pretreatment with DPI for 30 min led to a prominent retention of the IR-PCL $\gamma$ 1 complex despite the presence of bpV(phen) (see figures 3E and 3F). The data support the model whereby plasma membrane NADPH oxidase activity plays a key role in the regulation of protein-protein interaction of redox-sensitive signaling molecules, such as PCL $\gamma$ 1 and the IR.

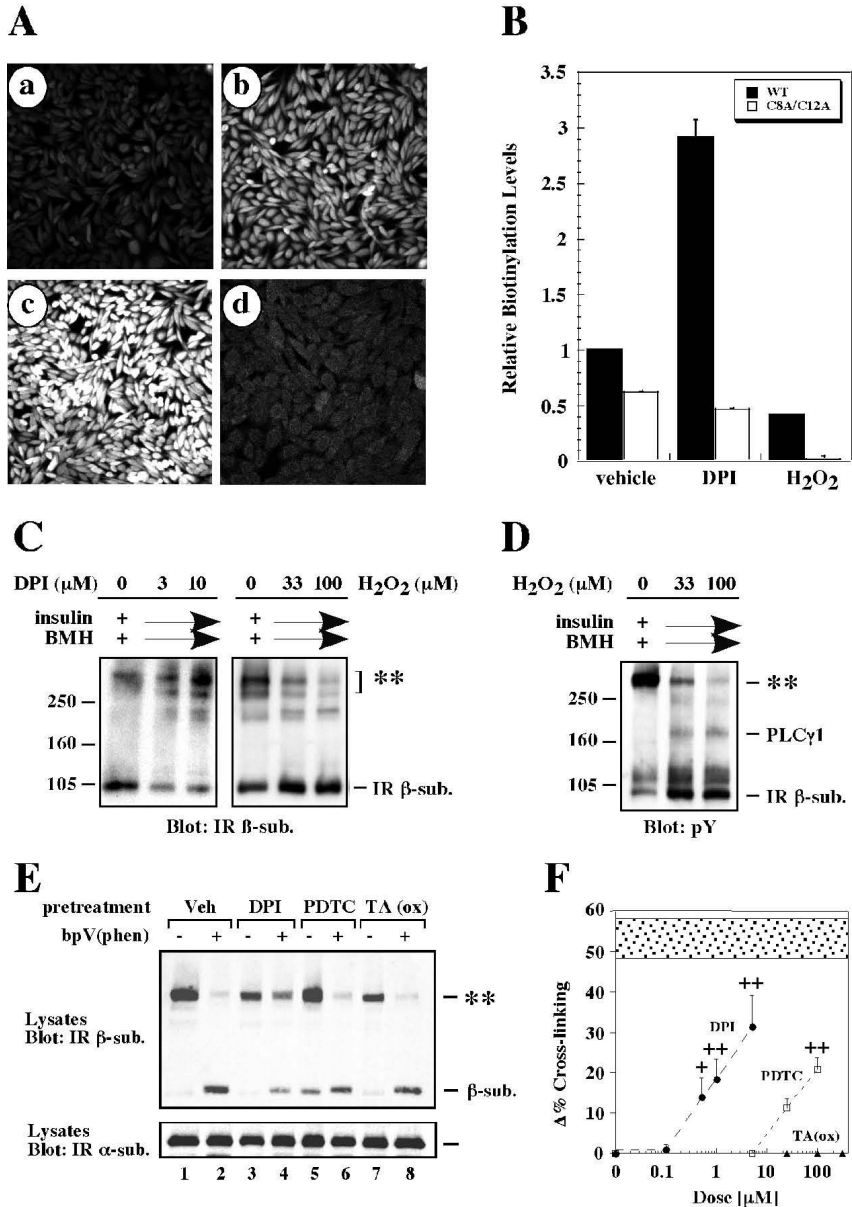
## DISCUSSION

In our previous work, we have shown that

FIGURE 3. (*facing page*) The effect of redox regulation on PCL $\gamma$ 1 thiol reactivity and its BMH-mediated covalent association with the IR. A) Production of  $H_2O_2$  in CHO-IR cells in response to various stimuli. Serum-starved CHO-IR cells were left untreated (a) or treated for 30 min with 100 nM insulin (b), or 5  $\mu$ M DPI for 20 min prior to the addition of insulin (d). Cells were then incubated with 4  $\mu$ M DCF for an additional 20 min in the dark. As control, exogenous  $H_2O_2$  (100  $\mu$ M, panel c) was added for 30 min prior to the addition of DCF. Intracellular  $H_2O_2$  production was detected by fluorescence of the indicator dye. B) Serum-starved CHO-IR cells expressing the wild-type PCL $\gamma$ 1 or C8A/C12A mutant were incubated with vehicle, 5  $\mu$ M DPI or 100  $\mu$ M  $H_2O_2$  for 30 min followed by thiol-specific biotinylation reaction with MBB. Anti-PLC $\gamma$ 1 immunoprecipitations were prepared, and the biotin incorporation in PCL $\gamma$ 1 quantitated by densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). A value of 1.0 was assigned to the biotin/PCL $\gamma$ 1 ratio of untreated cells expressing the wild-type PCL $\gamma$ 1. Bars are averages  $\pm$  range of two independent experiments. C, D) Thiol-specific crosslinking of PCL $\gamma$ 1 with IR in intact CHO-IR cells. E) Following a 30-min preincubation in the presence of vehicle, DPI (5  $\mu$ M), PDTC (25  $\mu$ M) or thiocetic acid (TA; 100  $\mu$ M), CHO-IR cells were treated without (–) or with (+) 20  $\mu$ M bpV(phen) for 1 h after which insulin (100 nM) was added for 10 min. Cells were then subjected to BMH crosslinking as described under Methods. Western blot analysis of cell lysates was performed with anti-IR  $\beta$ -subunit (upper panel) followed by immunodetection of IR  $\alpha$ -subunit (lower panel). F) Similar experiment as in E was performed, whereby various doses of DPI (0.1, 0.5, 1 and 5  $\mu$ M), PDTC (5, 25, 100  $\mu$ M) or TA (25, 100, 250  $\mu$ M) were used. Results are expressed as the means  $\pm$  SEM of three experiments, where the residual crosslinking signal elicited by bpV(phen) alone was subtracted from the signal obtained following preincubation with antioxidants. \*\* denotes the BMH-induced covalent complex between the IR  $\beta$ -subunit and PCL $\gamma$ 1. +, ++  $P < 0.05$  and  $< 0.01$ , respectively, compared with bpV(phen) alone.

PCL $\gamma$ 1 covalently binds to the activated IR upon addition of BMH to insulin-stimulated CHO-IR cells (Kwon *et al.*, 2003). Deletion of the juxtamembrane NPEY motif or truncation of the carboxyl 43 amino acids of the IR did not inhibit this process (Bernier *et al.*, 2004), suggesting that the association of PCL $\gamma$ 1 with the IR occurs at a region/motif that differs from that required by the well-known effectors of insulin action (Kharitononkov *et al.*, 1995; Sawka-Vervelle *et al.*, 1996; O'Neill *et al.*, 1996; Kasus-Jacobi *et al.*, 1998). It is generally accepted that intracellular redox plays an important role in the modulation of insulin action. The results of our study document an





active role of a discrete population of Cys-SH moieties in mediating IR-PCL $\gamma$ 1 covalent association in response to BMH, in particular those present in the NH<sub>2</sub>-terminus of PCL $\gamma$ 1. We approached their characterization by ectopically expressing wild-type, truncated ver-

sions and point-mutant forms of PCL $\gamma$ 1 proteins in CHO-IR cells.

Ectopic expression of PCL $\gamma$ 1 N-terminal fragment, 304N (which contains PH domain and EF-hands), was described as having great potency in interfering with endogenous IR-

PCL $\gamma$ 1 interaction (Kwon *et al.*, 2003). Our present data show that 304N contains cysteines that are reactive to cell redox modifiers. Substitution of PCL $\gamma$ 1 at Cys-8 and -12, but not those present in the PH-EF-hands, abrogated responsiveness to BMH while reducing thiol-alkylation with MBB by only 60%, which could indicate the presence of additional reactive Cys-SH moieties at a site (or sites) distant from the N-terminus of PCL $\gamma$ 1. By extension, we propose that these distant Cys residues do not play a role in BMH-mediated covalent binding of PCL $\gamma$ 1 to the IR, which relies instead on the close proximity of the N-terminus PCL $\gamma$ 1 thiol(s) with Cys981 of the human IR. We have recently observed that cell stimulation with insulin led to the detection of both the IR and activated, tyrosine-phosphorylated form of PCL $\gamma$ 1 in subdomains of the plasma membrane known as lipid rafts (Kwon *et al.*, in preparation). Rafts are enriched in actin filaments and have been proposed to serve several important functions, including the preassembly of signaling proteins onto a cytoskeletal scaffold (Whitehead *et al.*, 2000). Interestingly, reactive cysteine moieties are frequent sites for the posttranslational addition of lipids to integral membrane and membrane-associated proteins. These acylation reactions have been shown to be required for optimal targeting and proper function of several families of signaling molecules at the plasma membrane (Resh, 2004). Our present data are the first to document the presence of oxidation-sensitive nucleophilic thiols in PCL $\gamma$ 1; whether lipid modification of PCL $\gamma$ 1 is required for its targeting to rafts microdomains and tyrosine phosphorylation following cell activation by insulin is unclear. Such thioester bond may be transitory and have transient regulatory effects on the protein function. Experiments are currently underway to fully characterize the importance of a cell's redox system in controlling the targeting and cellular function of PCL $\gamma$ 1.

We found that the inhibition of cellular NADPH oxidases by DPI was the most effective approach against bpV(phen)-mediated decrease in BMH-dependant IR-PCL $\gamma$ 1 cross-linking. It is thought that bpV(phen) can induce superoxide anion production as a result of translocation and phosphorylation of the 47-kDa protein component of the NADPH oxidase at the plasma membrane where the catalytic flavocytochrome b resides (Yanase *et al.*, 1999). Herein, the increase in intracellular H<sub>2</sub>O<sub>2</sub> production with bpV(phen) in CHO-IR cells (data not shown) is likely to promote oxidative thiol modification on either the IR, PCL $\gamma$ 1 or both proteins, thus impacting on their reactivity toward BMH. Components of the NADPH oxidase are known to be localized on the cytoplasmic surface of the plasma membrane through association with the actin cytoskeleton (Woodman *et al.*, 1991; el Benna *et al.*, 1994; Buul *et al.*, 2005). Similar to the components of this oxidase, PCL $\gamma$ 1 binds to elements of the cytoskeleton that directly appose the plasma membrane of the cell (Chou *et al.*, 2002). These results support the view that insulin-mediated PCL $\gamma$ 1 signal transduction involves an oxidation-sensitive pathway through activation of NADPH oxidase at the plasma membrane.

Changes in cellular redox are known to occur with aging, diabetes and other metabolic conditions (Browlee, 2001). The consequences of oxidative damage include altered cell signaling, proliferation and apoptosis. We believe that PCL $\gamma$ 1 is target of such oxidative attack due to the presence of reactive cysteine residues in its N-terminal domain. PCL $\gamma$ 1 binding to the IR has been associated with upregulation of the Ras/ERK pathway in response to acute insulin stimulation (Kwon *et al.*, 2003). Better knowledge of the implications of redox alterations will open new opportunities into the understanding of PCL $\gamma$ 1 signaling and provide a new insight into the ways this enzyme exerts the pleiotropic ac-

tions of insulin in normal and pathophysiological states.

## REFERENCES

- BERNIER, M.; HE, H. J.; KWON, Y. K.; JANG, H. J. (2004). «The roles of phospholipase C- $\gamma$ 1 and actin-binding protein filamin A in signal transduction of the insulin receptor». *Vitam. Horm.*, 69: 221-247.
- BERNIER, M.; NADIV, O.; KOLE, H. K. (1995). «Thiol-specific biotinylation of the insulin receptor in permeabilized cells enhances receptor function». *Biochemistry*, 34: 8357-8364.
- BEVAN, A. P.; DRAKE, P. G.; YALE, J. F.; SHAVER, A.; POSNER, B. I. (1995). «Peroxovanadium compounds: biological actions and mechanism of insulin-mimesis». *Mol. Cell. Biochem.*, 153: 49-58.
- BIANCHINI, L.; NANDA, A.; WASAN, S.; GRINSTEIN, S. (1994). «Activation of multiple pH-regulatory pathways in granulocytes by a phosphotyrosine phosphatase antagonist». *Biochem. J.*, 301: 539-544.
- BROWNLEE, M. (2001). «Biochemistry and molecular cell biology of diabetic complications». *Nature*, 414: 813-820.
- BUUL, J. D. VAN; FERNANDEZ-BORJA, M.; ANTHONY, E. C.; HORDIJK, P. L. (2005). «Expression and localization of NOX2 and NOX4 in primary human endothelial cells». *Antioxid. Redox Signal.*, 7: 308-317.
- CHOU, J.; STOLZ, D. B.; BURKE, N. A.; WATKINS, S. C.; WELLS, A. (2002). «Distribution of gelsolin and phosphoinositid 4,5-bisphosphate in lamellipodia during EGF-induced motility». *Int. J. Biochem. Cell Biol.*, 34: 776-790.
- EICHHORN, J.; KAYALI, A. G.; AUSTIN, D. A.; WEBSTER, N. J. (2001). «Insulin activates phospholipase C- $\gamma$ 1 via a PI-3 kinase dependent mechanism in 3T3-L1 adipocytes». *Biochem. Biophys. Res. Commun.*, 282: 615-620.
- EICHHORN, J.; KAYALI, A. G.; RESOR, L.; AUSTIN, D. A.; ROSE, D. W.; WEBSTER, N. J. (2002). «PCL $\gamma$ 1 enzyme activity is required for insulin-induced DNA synthesis». *Endocrinology*, 143: 655-664.
- EL BENNA, J.; RUEDI, J. M.; BABIOR B. M. (1994). «Cytosolic guanine nucleotide-binding protein Rac2 operates in vivo as a component of the neutrophil respiratory burst oxidase. Transfer of Rac2 and the cytosolic oxidase components p47phox and p67phox to the submembranous actin cytoskeleton during oxidase activation». *J. Biol. Chem.*, 269: 6729-6734.
- FINKEL, T. (2003). «Oxidant signals and oxidative stress». *Curr. Opin. Cell Biol.*, 15: 247-254.
- GARANT, M.; KOLE, S.; MAKSIMOVA, E.; BERNIER, M. (1999). «Reversible change in thiol redox status of the insulin receptor  $\alpha$ -subunit in intact cells». *Biochemistry*, 38: 5896-5904.
- GARANT, M. J.; MAKSIMOVA, E.; MONTROSE-RAFIZADEH, C.; LEE-KWON, W.; KOLE, S.; BERNIER, M. (2000). «Cysteine 981 of the human insulin receptor is required for covalent cross-linking between  $\beta$ -subunit and a thiol-reactive membrane-associated protein». *Biochemistry*, 39: 7178-7187.
- JI, Q. S.; WINNIER, G. E.; NISWENDER, K. D.; HORSTMAN, D.; WISDOM, R.; MAGNUSON, M. A.; CARPENTER, G. (1997). «Essential role of the tyrosine kinase substrate phospholipase C- $\gamma$ 1 in mammalian growth and development». *Proc. Natl. Acad. Sci. USA*, 94: 2999-3003.
- KASUS-JACOBI, A.; PERDEREAU, D.; AUZAN, C.; CLAUSNER, E.; OBERGHEN, E. VAN; MAUVAIS-JARVIS, F.; GIRARD, J.; BURNOL, A. F. (1998). «Identification of the rat adapter Grb14 as an inhibitor of insulin actions». *J. Biol. Chem.*, 273: 26026-26035.
- KHARITONENKOV, A.; SCHNEKENBURGER, J.; CHEN, Z.; KNYAZEV, P.; ALI, S.; ZWICK, E.; WHITE, M. F.; ULLRICH, A. (1995). «Adapter function of protein-tyrosine phosphatase 1D in insulin receptor/insulin receptor substrate-1 interaction». *J. Biol. Chem.*, 270: 29189-29193.
- KWON, Y. K.; JANG, H. J.; KOLE, S.; HE, H. J.; BERNIER, M. (2003). «Role of the pleckstrin homology domain of PCL $\gamma$ 1 in its interaction with the insulin receptor». *J. Cell Biol.*, 163: 375-384.
- LORENZO, M.; TERUEL, T.; HERNANDEZ, R.; KAYALI, A. G.; WEBSTER, N. J. (2002). «PLC $\gamma$  participates in insulin stimulation of glucose uptake through activation of PKC $\zeta$  in brown adipocytes». *Exp. Cell Res.*, 278: 146-157.
- MAHADEV, K.; WU, X.; ZILBERING, A.; ZHU, L.; LAWRENCE, J. T.; GOLDSTEIN, B. J. (2001). «Hydrogen peroxide generated during cellular insulin stimulation is integral to activation of the distal insulin signaling cascade in 3T3-L1 adipocytes». *J. Biol. Chem.*, 276: 48662-48669.
- O'NEILL, T. J.; ROSE, D. W.; PILLAY, T. S.; HOTTA, K.; OLEFSKY, J. M.; GUSTAFSON, T. A. (1996). «Interaction of a GRB-IR splice variant (a human GRB10 homolog) with the insulin and insulin-like growth factor I receptors. Evidence for a role in mitogenic signaling». *J. Biol. Chem.*, 271: 22506-22513.
- RESH, M. D. (2004). «Membrane targeting of lipid modified signal transduction proteins». *Subcell. Biochem.*, 37: 217-232.
- RHEE, S. G. (2001). «Regulation of phosphoinositide-specific phospholipase C». *Annu. Rev. Biochem.*, 70: 281-312.
- ROY, S.; SEN, C. K.; TRITSCHLER, H. J.; PACKER, L. (1997). «Modulation of cellular reducing equivalent homeostasis by  $\alpha$ -lipoic acid. Mechanisms and implications for diabetes and ischemic injury». *Biochem. Pharmacol.*, 53: 393-399.
- SALTIEL, A. R.; PESSIN, J. E. (2002). «Insulin signaling pathways in time and space». *Trends Cell Biol.*, 12: 65-71.
- SAWKA-VERHELLE, D.; TARTARE-DECKER, S.; WHITE, M. F.; OBERGHEN, E. VAN (1996). «Insulin receptor substrate-2 binds to the insulin receptor through its phosphotyrosine-binding domain and through a newly identified

- domain comprising amino acids 591-786». *J. Biol. Chem.*, 271: 5980-5983.
- TELTING, D.; SMEETS, R. L.; WILLEMS, P. H.; ZON, G. C. VAN DER; FRANKHUIZEN, W. S.; MAASSEN, J. A. (1999). «The insulin receptor tyrosine kinase domain in a chimaeric epidermal growth factor-insulin receptor generates  $Ca^{2+}$  signals through the PLC- $\gamma$ 1 pathway». *Biochim. Biophys. Acta*, 1431: 421-432.
- WHITEHEAD, J. P.; CLARK, S. F.; URSO, B.; JAMES, D. E. (2000). «Signalling through the insulin receptor». *Curr. Opin. Cell Biol.*, 12: 222-228.
- WOODMAN, R. C.; RUEDI, J. M.; JESAITIS, A. J.; OKAMURA, N.; QUINN, M. T.; SMITH, R. M.; CURNUTTE, J. T.; BABIOR, B. M. (1991). «Respiratory burst oxidase and three of four oxidase-related polypeptides are associated with the cytoskeleton of human neutrophils». *J. Clin. Invest.*, 87: 1345-1351.
- YAMAGUCHI, M.; OISHI, H.; ARAKI, S.; SAEKI, S.; YAMANE, H.; OKAMURA, N.; ISHIBASHI, S. (1995). «Respiratory burst and tyrosine phosphorylation by vanadate». *Arch. Biochem. Biophys.*, 323: 382-386.
- YANAME, H.; FUKUNAGA, T.; NIGORIKAWA, K.; OKAMURA, N.; ISHIBASHI, S. (1999). «Pervanadate activates NADPH oxidase via protein kinase C-independent phosphorylation of p47-phox». *Arch. Biochem. Biophys.*, 361: 1-6.