Modification and Activation of Ras Proteins by Electrophilic Prostanoids with Different Structure are Site-Selective†

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Received November 18, 2006; Revised Manuscript Received March 29, 2007

ABSTRACT: Cyclopentenone prostanoids (cyP) arise as important modulators of inflammation and cell proliferation. Although their physiological significance has not been fully elucidated, their potent biological effects have spurred their study as leads for the development of therapeutic agents. A key determinant of cyP action is their ability to bind to thiol groups in proteins or in glutathione through Michael addition. Even though several protein targets for cyP addition have been identified, little is known about the structural determinants from the protein or the cyP that drive this modification. The results herein presented provide the first evidence that cyP with different structures target distinct thiol sites in a protein molecule, namely, H-Ras. Whereas 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and Δ^{12}-PGJ_2 preferentially target the C-terminal region containing cysteines 181 and 184, PGA_1 and 8-iso-PGA_1 bind mainly to cysteine 118, located in the GTP-binding motif. The biological counterparts of this specificity are the site-selective modification and activation of H-Ras in cells and the differential interaction of cyP with H, N, and K-Ras proteins. Cysteine 184 is unique to H-Ras, whereas cysteine 118 is present in the three Ras homologues. Consistent with this, PGA_1 binds to and activates H-, N-, and K-Ras, thus differing from the preferential interaction of 15d-PGJ_2 with H-Ras. These results put forward the possibility of influencing the selectivity of cyP-protein addition by modifying cyP structure. Furthermore, they may open new avenues for the development of cyP-based drugs.

Prostanoids with cyclopentenone structure (cyP[1]) are endogenous electrophilic lipids that exert varied biological functions through multiple mechanisms. Cyclopentenone isoprostanes and prostaglandins arise from the free radical-induced peroxidation of arachidonic acid (*1*) and the dehydration of prostaglandins (*2*, *3*), respectively. The organic synthesis of these compounds (*4*, *5*) has allowed one to substantiate their potent biological effects, which include antiviral and antiproliferative activities. CyP possess an α,β-unsaturated carbonyl group in the cyclopentane ring that favors the formation of Michael adducts with thiol groups in proteins or in glutathione (*4*, *6*). This mechanism is responsible for many of the biological effects of these compounds (*7*, *8*). Recent evidence indicates that cyP may play dual roles, eliciting a cytoprotective response at low concentrations and inducing cytotoxicity at higher levels (*9*). Protective effects of cyP have been encountered in animal models of tissue injury, ischemia/reperfusion, septic shock, and inflammation (*10*−*12*). However, cyP induce cytotoxicity in various cancer cell lines (*13*, *14*). Therefore, the potential use of cyP as leads for the development of antiinflammatory or antitumoral agents has encouraged the identification of protein targets for the addition of cyP (*15*, *16*). This could provide new therapeutic targets and help in the design of cyclopentenone-based drugs with improved selectivity or potency (*17*−*19*).

Ras proteins are crucial players in signaling processes controlling cell proliferation and differentiation. The switch between their active GTP-bound and inactive GDP-bound state is narrowly regulated by a complex interplay of nucleotide exchange factors and GTPase-activating proteins. Mammalian cells contain three Ras homologues, H-, N-, and K-Ras, the latter having two isoforms, K-Ras-4A and K-Ras-4B (*20*). Because Ras proteins share a high degree of sequence homology, it was long assumed that they played nearly redundant roles. However, in recent years, pharmacological and genetic evidence has made clear that the three Ras proteins play specific roles that arise from Ras type-dependent subcellular localization, regulation, and utilization of effector molecules (*21*−*25*). These differences are due in

† This work was supported by grants from Ministerio de Educación y Ciencia of Spain SAF2003-03713 and SAF2006-03489 to D.P.-S., SAF2003-02604, SAF2006-04247, and RETICS in Cancer from Instituto de Salud Carlos III to J.M.R., and BM04/179-1 and BM04/179-2 from Fundación la Caixa to D.P.-S. and J.M.R., respectively. J.G. has been supported by the CSIC I3P program (Fondo Social Europeo).

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[1] Abbreviations: CyP, cyclopentenone prostanoid(s); PG, prostaglandin; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; MALDI-TOF, matrix assisted laser desorption ionization-time-of-flight; MS, mass spectrometry; wt, wild type; HRP, horseradish peroxidase; RBD, Ras-binding domain of Raf; DMBA, 7,12-dimethylbenz[*a*]anthracene; TPA, tetradecanoylphorbol acetate.

10.1021/bi602389p CCC: $37.00
Published on Web 05/10/2007

part to the structural features of the hypervariable C-terminal regions, which are the site for Ras type-dependent post-translational modifications that provide additional levels of regulation (*23*, *26*). All Ras proteins are post-translationally modified by the isoprenylation of cysteine residues present in the carboxyl terminal CAAX motif (*27*). This is followed by proteolysis of the three amino acids distal to the isoprenylated cysteine and methylation of its newly exposed carboxyl group (*28*). These modifications increase the hydrophobicity of the proteins facilitating their interaction with membranes (*29*). Remarkably, H-Ras and N-Ras, but not K-Ras, possess additional cysteines (C181 and C184 in human H-Ras and C181 in N-Ras), which are sites for reversible palmitoylation (*27*, *30*, *31*).

We have recently described that H-Ras can be a target for the addition of the cyP 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2). This effect is associated with an activation of H-Ras-dependent pathways, increased proliferation in NIH-3T3 fibroblasts (*32*), and protection from apoptosis in MCA3D keratinocytes (*33*). The biological correlation of these effects is a significant enhancement of the carcinogenesis in the skin of mice elicited by the classical treatment with DMBA/TPA (*33*). Thus, Ras proteins constitute a relevant model for the study of the structural requirements and functional consequences of cyP addition.

In this work, we have studied the ability of a varied array of cyP to covalently modify Ras proteins. Our results provide the first evidence showing that the structure of the prostanoid directs adduct formation to distinct cysteine residues on the protein molecule, thus favoring the differential modification of Ras proteins by diverse cyP.

EXPERIMENTAL PROCEDURES

Materials. The prostanoids used throughout this study were from Cayman Chemical (Ann Arbor, MI). Biotinylated 15d-PGJ_2 was prepared as previously described (*34*). Human recombinant H-Ras was from Calbiochem-Novabiochem (San Diego, CA). Anti-HA (sc-7392) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA), anti-AU5 monoclonal antibody was from Berkeley Antibody Company (Berkeley, CA), anti-pan Ras (Ab-3) was from Merck, and EGF was purchased from Sigma-Aldrich (St. Louis, MO).

DNA Constructs. The plasmids pCEFL-KZ-HA, pCEFL-KZ-AU5, pCEFL-AU5-H-Ras wt, pCEFL-AU5-N-Ras wt, pCEFL-AU5-K-Ras4B wt, pCEFL-KZ-AU5-H-Ras C118S, pCEFL-KZ-AU5-H-Ras C184S, pCEFL-KZ-HA-H-Ras wt, pCEFL-KZ-HA-N-Ras wt, pCEFL-HA-K-Ras4B wt, pCEFL-KZ-HA-H-Ras C118S, and pCEFL-KZ-HA-H-Ras C184S were previously described (*25*, *32*).

Cell Culture and Transfections. COS-7 and HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Transient transfections were performed with Lipofectamine 2000 (Invitrogen) or Jet-Pei (Polyplus-Transfection, Illkirch, France) according to the instructions of the manufacturer. For treatment with prostanoids or their biotinylated analogues, cells were incubated in the absence of serum. Potential toxicity of the reagents used was evaluated by trypan blue exclusion. According to these criteria, cell viability was above 90% under all experimental conditions studied.

Analysis of the Interaction between cyP and H-Ras in Vitro. H-Ras at 5 μM final concentration was incubated in 20 mM Tris-HCl at pH 7.0, 45 mM NaCl, 5 mM $MgCl_2$, 0.1 mM DTT, and 1.4% glycerol. The various compounds were added in DMSO at 50 μM. After a 2 h of incubation, mixtures were prepared for analysis with ZipTip C18 before or after digestion with trypsin for 4 h at 37 °C. MALDI-TOF mass spectrometry analysis of intact H-Ras proteins and tryptic digests was performed essentially as described (*34*). For intact Ras proteins, the matrix used was a saturated solution of sinapinic acid in acetonitrile/water (1:2) with 0.1% trifluoroacetic acid (TFA). Because of the substantial mass difference between the matrix (224 Da) and the various cyP used in this study (>300 Da), the interference between the protein−matrix adducts and the Ras−cyP complexes was negligible. Protein calibration standard II (Bruker Daltonics, Bremen, Germany) was used for external calibration, and samples were analyzed in the linear mode. For analysis of tryptic digests, the matrix was a saturated solution of α-cyano-4-hydroxycinnamic acid in acetonitrile/water (1:2) with 0.1% TFA, and the external calibration mixture contained angiotensin (*m*/*z* 1046.5), corticotrophin (*m*/*z* 2465.2), and the matrix (α-cyano-4-hydroxycinnamic acid, *m*/*z* 379) and samples were analyzed in the reflectron mode. The spectra were obtained in a BIFLEX III time-of-flight instrument (Bruker-Franzen Analytik, Bremen, FRG) operated in the positive mode. MALDI-TOF MS-MS analysis of selected peptides was performed using the MALDI-tandem time-of-flight mass spectrometer 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA) at the Proteomics Unit (Parque Científico, Madrid, Spain). Spectra were submitted to Mascot (Matrix Science, London) and BLAST database search routines for confirmation of results. Probability based MOWSE scores were calculated as $-10*\log(P)$, where P is the probability that the observed match is a random event. Scores were considered significant when $P < 0.05$.

Binding of Biotinylated CyP to Ras Proteins in Intact Cells. COS-7 cells transiently transfected with Ras plasmids were incubated with biotinylated cyP for 2 h. Cells were lysed in 50 mM Tris-HCl at pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM 2-mercaptoethanol, and 0.5% SDS containing protease inhibitors (2 μg/mL each of leupeptin, aprotinin, and pepstatin, and 1.3 mM ABMSF), and incorporation of biotinylated cyP into Ras proteins was assessed by Western blot with HRP-streptavidin, essentially as described (*32*).

Bacterial Expression of Fusion Proteins. The GST-fusion protein, GST-RBD (containing the Ras binding domain of Raf fused to GST), was purified following the method previously described (*35*) from *E. coli* Bl21 (DE3) harboring the corresponding plasmid to express the protein.

Raf Ras Binding Domain Pull-Down. Levels of active Ras, that is, able to interact with Raf, were estimated by pull-down assays using glutathione-sepharose-GST-RBD beads and immunoblotting with specific antibodies, essentially as described (*32*). Cells were stimulated for 15 min with 30% fetal bovine serum, 100 nM EGF, 3 μM 15d-PGJ_2, or 10 μM PGA_1, and lysed in cold lysis buffer containing 25 mM HEPES at pH 7.5, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM sodium orthovanadate (Na_3VO_4), 25 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/mL each of leupeptin, aprotinin, pepstatin A, and trypsin

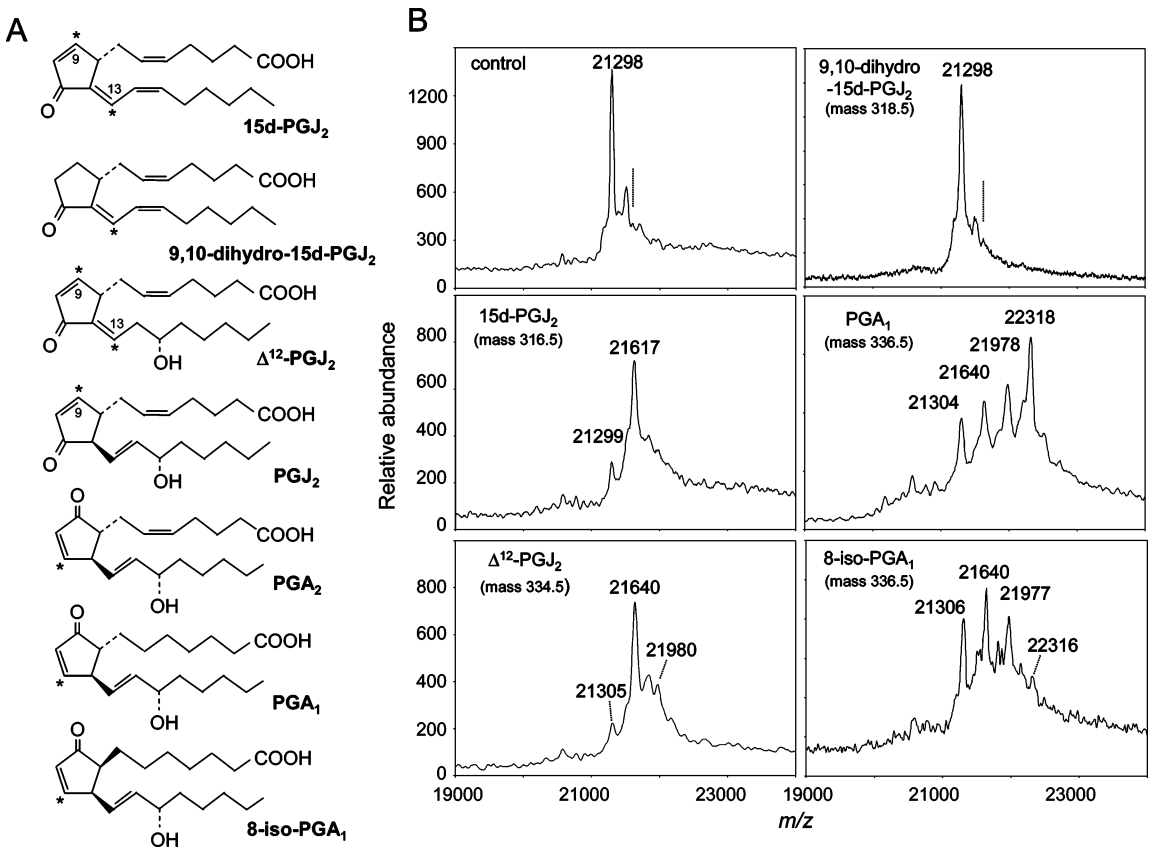

FIGURE 1: MALDI-TOF MS analysis of the interaction of various electrophilic compounds with H-Ras proteins. (A) Structures of the compounds used in this study. (B) H-Ras (5 μM) was incubated for 1 h at rt in the presence of the various compounds at 50 μM. After purification on ZipTip, H-Ras was analyzed by MALDI-TOF MS. Spectra shown are representative of at least two assays per experimental condition. The dotted line in control and 9,10-dihydro-PGJ_2 indicates the position where the cyP-H-Ras adduct appears in the 15d-PGJ_2-treated sample.

inhibitor. Post-nuclear supernatants were incubated with GST-RBD on glutathione-sepharose beads and analyzed as described previously (*35*).

Statistical Analysis. The data are shown as the mean ± SE of at least three experiments. Statistical significance was estimated with the Student's *t* test for unpaired observations. A *p* value of less than 0.05 was considered significant.

RESULTS

Differential Modification of H-Ras Proteins by Electrophilic Eicosanoids with Diverse Structure. H-Ras proteins can be modified by cyP (*32*). In this study, we used a panel of cyP, the structures of which are depicted in Figure 1A, to explore their ability to covalently bind to Ras proteins. H-Ras incubated in the presence of vehicle gave a peak of *m*/*z* 21298 and the matrix adduct peak (+224) (Figure 1B). Incubation of H-Ras with the different compounds resulted in the appearance of diverse modifications. Of the compounds studied, 15d-PGJ_2 and Δ^{12}-PGJ_2 formed one major adduct with H-Ras, whose *m*/*z* corresponded to the incorporation of one cyP molecule into H-Ras, thus indicating that these cyP bind preferentially to one residue of the protein (Figure 1B), although in the case of Δ^{12}-PGJ_2, a second adduct of lower relative abundance was detected, consistent with the addition of two cyP. In contrast, PGA_1 and 8-iso-PGA_1 clearly formed several adducts of masses compatible with the addition of one, two, or three molecules of cyP to H-Ras, thus showing that these compounds can modify several residues in H-Ras proteins. PGJ_2 and PGA_2 also formed several adducts (not shown), whereas the compound 9,10-dihydro-PGJ_2, an analogue of 15d-PGJ_2 that lacks the endocyclic double bond, showed a reduced capacity to bind to H-Ras proteins *in vitro* (Figure 1B).

Prostanoids with Different Structure Selectively Modify Distinct H-Ras Cysteine Residues in Vitro. To gain deeper insight into the differential modification of H-Ras by cyP with diverse structure, we analyzed control and cyP-modified H-Ras proteins by digestion with trypsin and MALDI-TOF MS (Figure 2). A detailed description of the peptide fingerprint of control Ras is provided in Supporting Information. The peptide fingerprints of H-Ras proteins modified by the various compounds showed several peptides whose *m*/*z* values were compatible with the presence of cyP adducts. Some of the putatively modified peptides are marked by asterisks in Figure 2, and a detailed list is given in Figure 3 (see below). The sites of modification that were detected mapped to the peptide fragment(s) located near the carboxyl terminal end of the protein containing cysteines 181 and 184, and/or to the peptide containing cysteine 118, which is located in the GTP binding motif (*36*). Interestingly, the relative intensities of the modified peptides were different depending on the cyP used. After treatment of H-Ras with 15d-PGJ_2 or Δ^{12}-PGJ_2, the most abundant modified species detected (a cluster of peptides of *m*/*z* 1979.2, 1995.2, and 2011.2 in the case of 15d-PGJ_2-treated H-Ras and peptide 1997.2 in the case of Δ^{12}-PGJ_2-treated H-Ras) were consis-

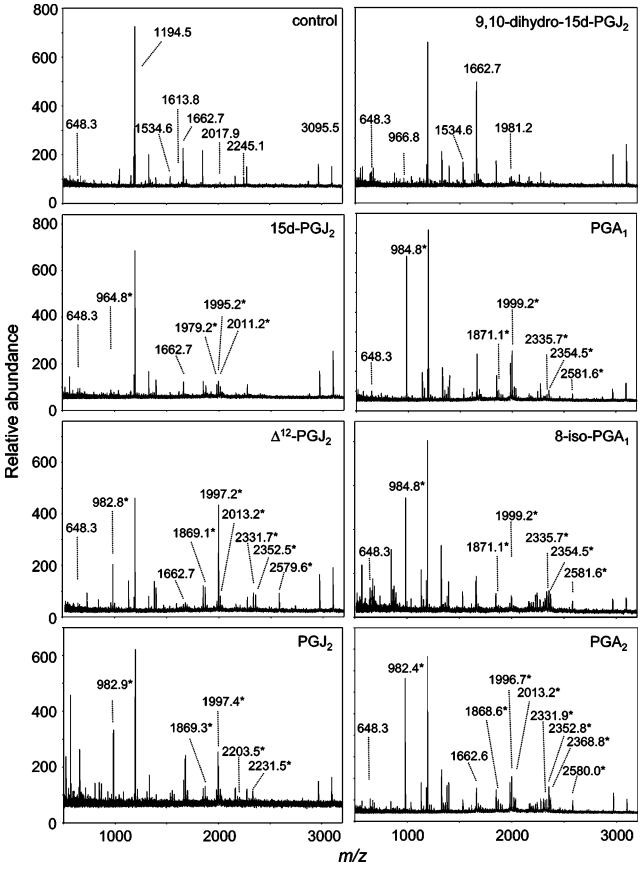

FIGURE 2: MALDI-TOF MS analysis of tryptic digests of cyP-modified H-Ras proteins. H-Ras incubated with the indicated prostanoids was subjected to digestion with trypsin, purification on ZipTip, and MALDI-TOF MS. The monoisotopic *m*/*z* values of some of the peptides detected are indicated. Major modified peaks appear around 960−990 and/or 2000 on the *x*-axis scale. Peptide fingerprints shown are representative of at least two assays per experimental condition.

tent with the addition of the cyP to the peptide 170−185, which contains cysteines 181 and 184. In the tryptic digest of Δ^{12}-PGJ_2-treated H-Ras, some peaks were detected (2203.6 and 2331.7) that were compatible with the incorporation of two cyP molecules into peptides containing cysteines 181 and 184. Treatment of H-Ras with PGA_1, 8-iso-PGA_1, PGA_2, or PGJ_2 gave rise to the appearance of several peptides consistent with the modification of cysteines 118, 181, and /or 184. In the case of PGA_2, PGA_1, and 8-iso-PGA_1, the peptides corresponding to the modification of cysteine 118 (*m*/*z* 984.8 for PGA_1 and 8-iso-PGA_1, and 982.4 for PGA_2) constituted the major species detected. The qualitative and quantitative differences observed in the peptide fingerprints of H-Ras proteins modified by the various cyP are summarized in Figure 3. The data shown in Figure 3A list the *m*/*z* and theoretical positions in the sequence of H-Ras of potentially modified peptides for which compatible peaks were observed in the tryptic digests of the various cyP-modified proteins. The relative intensities of the putatively modified peptides containing cysteine 118 plus one cyP molecule, cysteine 181 and 184 plus one cyP molecule, or cysteine 181 and 184 plus two cyP molecules, observed in every experimental condition, are represented in Figure 3B. This analysis clearly illustrates the site selectivity of the *in vitro* modification of H-Ras protein by cyP with different structure. The identity of the major modified peptides was confirmed by analysis of the corresponding peaks by MALDI-TOF-TOF MS-MS (Figure 4). The peptide of *m*/*z* 984.8 from PGA_1-treated H-Ras indeed corresponded to the tryptic peptide CDLAAR containing cysteine 118 (*m*/*z* 648. 3) plus one PGA_1 molecule. The mass increment of 336.5 was observed on the ions b2-H_2O, b3-H_2O, b4-H_2O, and y6, which indicates the modification of cysteine 118. The peptide of *m*/*z* 1997.2 from Δ^{12}-PGJ_2-treated H-Ras was confirmed to be the tryptic peptide KLNPPDESGPGCMSCK containing cysteines 181 and 184 (*m*/*z* 1662.6) plus one PG molecule. The mass increment of 334.5 was observed on ions y7−y15. In both cases, the ions corresponding to retro-Michael fragmentation of the modified peptides (*m*/*z* 648.3 and 1662.6, respectively) were observed in the MALDI-TOF-TOF MS-MS spectra. The identity of peptides of *m*/*z* 1979.2 and 1997.4 from 15d-PGJ_2- and PGJ_2-treated H-Ras, respectively, corresponding to the addition of one cyP molecule to peptide 170−185, was also confirmed by MS-MS analysis (data not shown).

Biotin-15d-PGJ_2 and Biotin-PGA_1 Differentially Modify H-Ras Proteins in Vitro and in Cells. The results shown above indicate that cyP with different structure preferentially bind to different cysteine residues in H-Ras *in vitro*. In order to explore the potential differential modification of H-Ras proteins by eicosanoids in biological systems, we used biotinylated analogues of two cyP that displayed widely divergent binding selectivity, namely, biotin-15d-PGJ_2 and biotin-PGA_1, both of which retain the cyclopentenone moiety (Figure 5A). We first confirmed that the reactivity of the biotinylated compounds toward H-Ras proteins *in vitro* was similar to that of non-biotinylated cyP. Biotin-15d-PGJ_2 (mass 626) formed a single adduct with H-Ras as detected by MALDI-TOF MS, whereas two or three adducts could be detected with biotin-PGA_1 (mass 661) (Figure 5B). The main target peptides were the same for biotinylated and non-biotinylated cyP (not shown). In addition, we assessed the binding of biotinylated cyP to H-Ras proteins by Western blot. As shown in Figure 5C, incubation of H-Ras with biotin-15d-PGJ_2 resulted in the appearance of a major modified band of reduced electrophoretical mobility with respect to the nonmodified protein. In contrast, modification by biotin-PGA_1 was characterized by the appearance of two clearly detectable biotinylated bands with higher apparent molecular mass than that of nonmodified H-Ras. These observations confirm the differential modification of H-Ras proteins by the two types of cyP. Next, we used these compounds to assess the binding of cyP to H-Ras in cells. We have previously reported that both biotin-15d-PGJ_2 and biotin-PGA_1 form stable covalent adducts with cellular proteins in intact cells, with biotin-15d-PGJ_2 being more effective (*37*). Therefore, for subsequent experiments, we used concentrations of biotin-15d-PGJ_2 and biotin-PGA_1, which labeled the total cellular protein with similar intensity, namely, 3 and 30 μM final concentrations, respectively (Figure 5D). Both biotinylated cyP bound to H-Ras proteins in intact COS7 cells (Figure 5E). Similar results were obtained in several cell types, including HeLa and NIH-3T3 cells (not shown).

Structural Requirements for the Interaction between H-Ras Proteins and cyP in Cells. We next assessed the sites of modification of H-Ras by the addition of biotinylated cyP in intact cells (Figure 6A). Interestingly, the binding of biotin-

A

Cysteine-containing peptides Position	*Theoretical m/z of peptides potentially modified by cyP for which compatible peptides were observed experimentally*							Sequence
	control	+15d-PGJ_2 (316.5)	+Δ^{12}-PGJ_2 (334.5)	+PGJ_2 (334.5)	+PGA_2 (334.5)	+PGA_1 (336.5)	+8-iso-PGA_1 (336.5)	
186-189	421.25	-	-	-	-	-	-	**C**VLS
118-123	648.31	964.81	982.81	982.81	982.81	984.81	984.81	**C**DLAAR
171-185	1534.63	-	1869.13	1869,13	1869,13	1871.13	1871.13	LNPPDESGPG**C**MS**C**K
170-185	1662.73	1979.23	1997.23	1997.43	1997.43	1999.23	1999.23	KLNPPDESGPG**C**MS**C**K
"		1995.23	2013.23	2013.23	2013.23	-	-	KLNPPDESGPG**C**MS**C**K (+O)
"		2011.23	2029.23	2029.23	2029.23	-	-	KLNPPDESGPG**C**MS**C**K (+2O)
171-185	1534.63	-	2203.63	2203.63	2203.63	-	-	LNPPDESGPG**C**MS**C**K (+2cyP)
170-185	1662.73	-	2331.73	2331.73	2331.73	2335.73	2335.73	KLNPPDESGPG**C**MS**C**K (+2cyP)
105-123	2017.97	-	2352.47	2352.47	2352.47	2354.47	2354.47	DSDDVPMVLVGNK**C**DLAAR
105-123	2017.97	-	-	-	2368.47	-	-	DSDDVPMVLVGNK**C**DLAAR (+O)
103-123	2245.13	-	2579.63	2579.63	2579.63	2581.63	2581.63	VKDSDDVPMVLVGNK**C**DLAA R

B

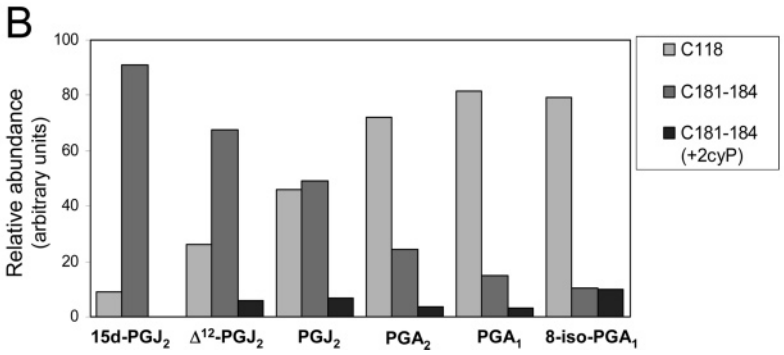

FIGURE 3: Differential modification of H-Ras cysteine residues by cyP *in vitro*. (A) Summary of the calculated *m*/*z* of peptides potentially modified by every cyP, their position in the sequence of H-Ras, and their sequence. For every experimental condition, only the values for which compatible peaks were observed experimentally in the tryptic digests of the various cyP-modified proteins are shown. Cysteine residues are depicted in bold. +O, *m*/*z* compatible with the addition of oxygen; +2cyP, *m*/*z* compatible with the addition of two cyP molecules. (B) Relative intensities of the modified peaks in H-Ras treated with diverse cyP. The intensities of the various modified peaks observed, corrected by the intensity of the non-adducted peptide 3095.5, are expressed as a percentage of the sum of the intensity of all modified peaks detected in every assay. Results shown are average values of at least two determinations. C118 and C181-184, peaks compatible with the addition of one cyP molecule to peptides containing cysteine 118 or cysteines 181 and 184, respectively; C181-184 (+2cyP), peaks compatible with the addition of two cyP molecules to peptides containing cysteines 181 and 184.

15d-PGJ_2 to H-Ras was strongly reduced by mutation of cysteine 184, whereas the mutation of cysteine 118 had no effect. Conversely, binding of biotin-PGA_1 to H-Ras was partially reduced by mutation of either cysteine 184 or cysteine 118. These observations indicate that biotinylated cyP also show a differential reactivity toward H-Ras cysteine residues in intact cells. We have previously reported that the modification of H-Ras proteins by 15d-PGJ_2 correlates with an increase in the proportion of Ras-GTP in intact cells (*32*). Here, we have compared the effect of 15d-PGJ_2 and PGA_1 on the levels of active Ras in HeLa cells transiently transfected with expression vectors coding for wild type or cysteine-to-serine mutant H-Ras proteins (Figure 6B). Both cyP elicited the activation of wild type H-Ras. However, they differed in their ability to activate H-Ras mutants. H-Ras activation associated with the treatment with PGA_1 was reduced by mutation of cysteine 118 (Figure 6B). A similar reduction was observed after the mutation of cysteines 118 and 184 (data not shown). Nevertheless, the C118S mutant was activated by 15d-PGJ_2 to similar levels compared to that of H-Ras wt (Figure 6B). In contrast, 15d-PGJ_2-elicited activation of the H-Ras C184S mutant, which possesses reduced capacity to incorporate 15d-PGJ_2 (Figure 6A), was near basal levels, according to our previously described observations (*32*). In contrast, this mutant was activated by PGA_1 to an extent close to that of H-Ras wt (Figure 6B).

Differential Selectivity of the Modification and Activation of H-, N-, and K-Ras Proteins by cyP. The results obtained thus far indicate that the presence of cysteine 184 is important for 15d-PGJ_2 to bind to H-Ras and elicit its activation; however, it is not essential for PGA_1-elicited modification, which can also take place at cysteine 118. It is interesting to note that cysteine 184 is unique to H-Ras, whereas cysteine 118 is common to the three Ras protein homologues. This raises the possibility that 15d-PGJ_2 could interact more specifically with H-Ras, whereas PGA_1 could affect H-, N-, and K-Ras-dependent pathways. Therefore, we explored the binding of biotinylated 15d-PGJ_2 and PGA_1 to the three Ras proteins transiently expressed in intact cells. We observed that biotin-15d-PGJ_2 preferentially bound to H-Ras, whereas biotin-PGA_1 showed less specificity (Figure 7A). We have previously reported that treatment with 15d-PGJ_2 selectively activates H-Ras in cells (*32*). Here, we have found that PGA_1 activates all Ras homologues to a similar extent (Figure 7B), thus supporting the relevance of cysteine 118 as a target site for PGA_1.

DISCUSSION

CyP may form covalent adducts with various cellular proteins, and this process plays an important role in their biological effects (*38*). Several studies have recently addressed the identification of the protein targets for cyP

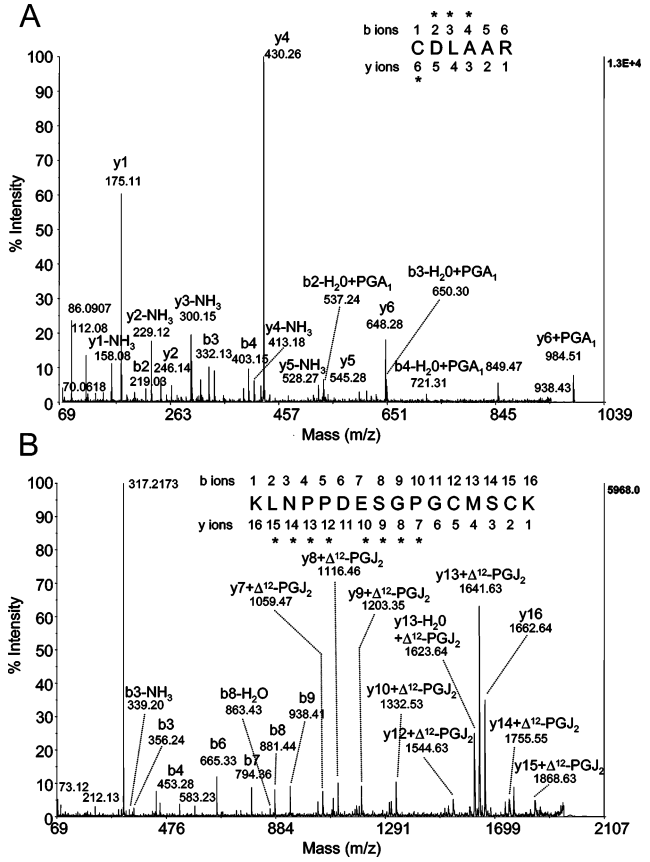

FIGURE 4: MALDI-TOF-TOF MS-MS analysis of the modified peptides. Peptides with *m/z* 984.8 from PGA_1-treated H-Ras (A) and 1997.2 from Δ^{12}-PGJ_2-treated H-Ras (B) were subjected to MS-MS analysis. The sequences of the peptides are given above the spectra. Asterisks denote the ions for which peaks compatible with the addition of the respective cyP are observed.

addition, either on an individual basis or through proteomic approaches (*15*, *16*, *39*, *40*). CyP targets include the transcription factors NF-κB and AP-1 (*34*, *39*), the redox-sensing protein Keap-1 (*41*), and the tumor suppressor LKB1 (*42*), among others. In spite of these findings, the structural determinants of either the protein or the cyP that favor this modification remain poorly understood. The observation that the pattern of proteins modified by biotin-15d-PGJ_2 is different from that modified by biotinylated iodoacetamide (*38*) indicates that cyP target a selective subset of cellular proteins with accessible thiol groups. Moreover, the 2D-electrophoresis pattern of proteins modified by biotin-15d-PGJ_2 does not completely overlap with that of biotin-PGA_1 targets (*37*), which brings forward the importance of cyP structure in the selectivity of protein modification. The results presented in this study show for the first time that cyP with different structure selectively modify distinct sites in an individual protein *in vitro* and in intact cells and suggest that this selectivity may have implications for signal transduction.

The structural determinants involved in the different behavior of cyP observed *in vitro* could be multiple. We observed that 15d-PGJ_2 and Δ^{12}-PGJ_2 bound preferentially to the C-terminus of H-Ras. These cyP present several remarkable features. First, both possess dienone structure, that is, they possess two electrophilic carbons, marked by asterisks in Figure 1A, through which they can form Michael adducts with two thiol groups. In fact, bis adducts of the

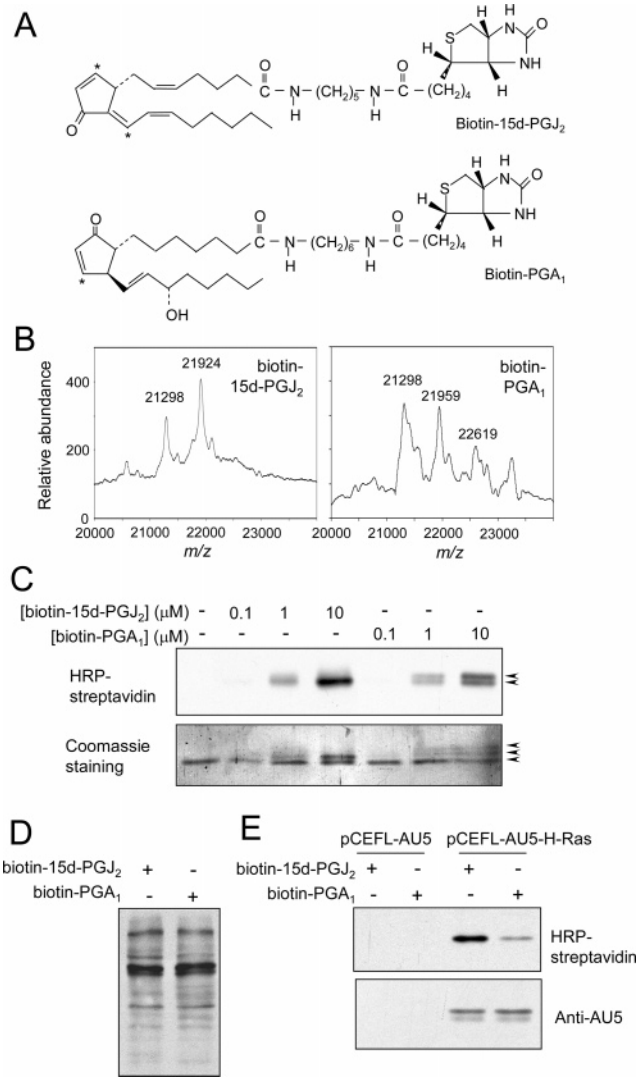

FIGURE 5: Modification of H-Ras proteins by biotinylated 15d-PGJ_2 and PGA_1. (A) Structures of the biotinylated derivatives of 15d-PGJ_2 and PGA_1. (B) MALDI-TOF MS analysis of H-Ras incubated with biotin-15d-PGJ_2 or biotin-PGA_1. (C) H-Ras treated as in B analyzed by SDS−PAGE and Western blot with HRP-streptavidin and ECL, after which the blot was stained with Coomassie. Arrowheads mark the position of the unmodified (lower) and modified (upper) H-Ras protein bands. Results are representative of three independent assays. (D) COS-7 cells incubated with 3 μM biotin-15d-PGJ_2 or 30 μM biotin-PGA_1 and the incorporation of the biotin label into cellular proteins assessed by SDS−PAGE and Western blot. (E) Cells transiently transfected with vector or with pECFL-AU5-H-Ras incubated with biotinylated cyP as in D and incorporation of the biotin label into H-Ras evidenced as described in the Experimental Procedures section. Levels of AU5-H-Ras were estimated by Western blot. The results are representative of four assays.

dienone 9-deoxy-Δ^9,Δ^{12}(E)-PGD_2 with GSH (*43*) and 15d-PGJ_2 with c-Jun (*34*) have been previously reported. The C-terminus of H-Ras possesses several cysteine residues in close proximity, whose presence could favor the binding of dienone prostaglandins at this site. That all ions from the MS/MS analysis of the 170−185 peptide from Δ^{12}-PGJ_2-treated H-Ras that showed a mass increment of 334, corresponding to the addition of one Δ^{12}-PGJ_2 molecule, contained cysteines 181 and 184 is noteworthy. Thus, the possibility exists that at least a portion of the modified peptide may represent the simultaneous addition of one PG molecule to both cysteines. In fact, this peptide did not show

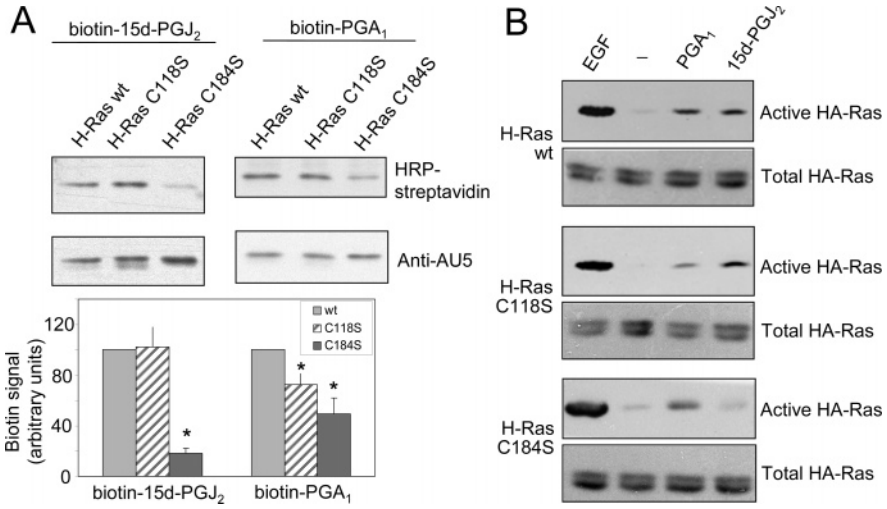

FIGURE 6: Modification and activation of H-Ras wt and mutants by cyP. (A) COS7 cells transiently transfected with the indicated plasmids were incubated in the presence of 3 μM biotin-15d-PGJ_2 or 30 μM biotin-PGA_1. Incorporation of biotinylated cyP into Ras wt, C118S, or C184S was assessed as specified in the legend for Figure 5. Levels of total H-Ras were assessed by Western blot with anti-AU5 antibody. ECL exposures were quantitated by image scanning. The ratios between the intensities of the biotin and the protein signal are expressed as the percentage of the values obtained for H-Ras wt. Results shown are average values ± SEM of four determinations (*$p < 0.05$ by *t*-test vs H-Ras wt). (B) Cells transiently transfected with the indicated plasmids were serum-starved for 18 h and then incubated with vehicle (−), 100 nM EGF, 3 μM 15d-PGJ_2, or 10 μM PGA_1. Ras in its active conformation (active Ras) was recovered from cell lysates by binding to immobilized GST fused to the Ras−GTP binding domain of Raf and detected by immunoblotting with anti-HA monoclonal antibody. The expression levels of the transfected constructs were assessed by immunoblotting of the cell extracts with anti-HA antibody (lower panels). The results shown are from a representative experiment. Similar results were obtained in four additional, separate experiments.

any further mass increment upon reduction with 10 mM DTT and alkylation with 50 mM iodoacetamide (data not shown), which suggests that it does not possess accessible cysteine residues. In contrast, PGA_2, PGA_1, and 8-iso-PGA_1 formed several adducts with H-Ras, with the preferential binding site apparently being cysteine 118. All these compounds are single enone cyP. The reactivity of single enone and dienone cyP toward thiol groups displays important differences, with dienone cyP forming adducts with thiols more rapidly. Moreover, adducts of dienone cyP with soluble thiols such as glutathione are less stable than those formed by single enone cyP (*6*). In addition to the differences in binding kinetics (*6*), the binding selectivity of cyP could also be related to the constrictions imposed by the exocyclic double bond to the geometry and flexibility of the lateral chains in the case of the dienones 15d-PGJ_2 or Δ^{12}-PGJ_2, compared to single enones.

CyP addition to H-Ras involves cysteine residues that can be modified by oxidants or that are sites for lipidation in cells. The four surface-exposed cysteine residues of H-Ras (cysteines 118, 181, 184, and 186) can be modified by oxidants *in vitro*, and extensive oxidative modifications occur also *in vivo* (*44*). Cysteine 186 is the site for isoprenylation. Cysteine 118 is the site for interaction with NO, which leads to increased nucleotide exchange and H-Ras activation (*45*), the mechanism of which has been recently studied in detail (*36*). Mutation of cysteine 118 to serine abolishes the activation of Ras in response to NO or superoxide anion (*46*); however, it does not disrupt the structure of the protein, nor alter its GTPase activity or GDP dissociation rate (*47*). Moreover, C118S H-Ras can be fully activated by serum (*32*). Cysteines 181 and 184 are sites for palmitoylation (*29*), a modification that finely controls the subcellular localization of H-Ras proteins (*31*). However, the contribution of the palmitoylation of these two cysteine residues to Ras localization and signaling is not equivalent (*48*). The C184S H-Ras mutant traffics to the plasma membrane in a manner similar to that of wild type H-Ras, whereas the C181S mutant localizes to the Golgi apparatus. The C184S H-Ras mutant has also been shown to efficiently recruit Raf and induce ERK activation (*48*). Here, we have shown that this mutant is fully activated by EGF. Therefore, it is not likely that the lack of activation of H-Ras C184S by 15d-PGJ_2 is due to alterations in the subcellular localization or in the interaction of this mutant protein with regulators. Cysteines 181 and/or 184 may be accessible for modification by electrophilic lipids in cells because H-Ras palmitoylation is a dynamic process (*30*). The palmitic acid linkage can be hydrolyzed enzymatically, and it is susceptible to thiol exchange (*44*). Moreover, palmitate turnover on H-Ras can be stimulated by some nitric oxide donors (*49*). Therefore, cyP addition to H-Ras may coexist or compete with oxidative modifications and/or palmitoylation.

In this study, we show a correlation between the ability of cyP to modify Ras proteins and an increase in the levels of active Ras, which indicates that cyP binding may cause Ras activation. However, other possibilities should be taken into account, including the formation of radicals or the occurrence of oxidative modifications, particularly in cells in which cyP may induce oxidative stress (*1*, *50*). Nevertheless, this mechanism is unlikely in the case of 15d-PGJ_2-induced activation, given the fact that mutation of cysteine 118, which is involved in the direct activation of H-Ras by reactive oxygen and nitrogen species (*46*), does not reduce activation by this cyP. Our results indicate that diverse cyP may preferentially modify and activate distinct Ras protein homologues. Because the site of cyP binding may be different depending on cyP structure and the Ras homologue, the mechanism of activation could be different in every situation. The binding of cyP to the C-terminal region of Ras could have an impact on Ras membrane association or on protein−protein interactions in which this region plays an important

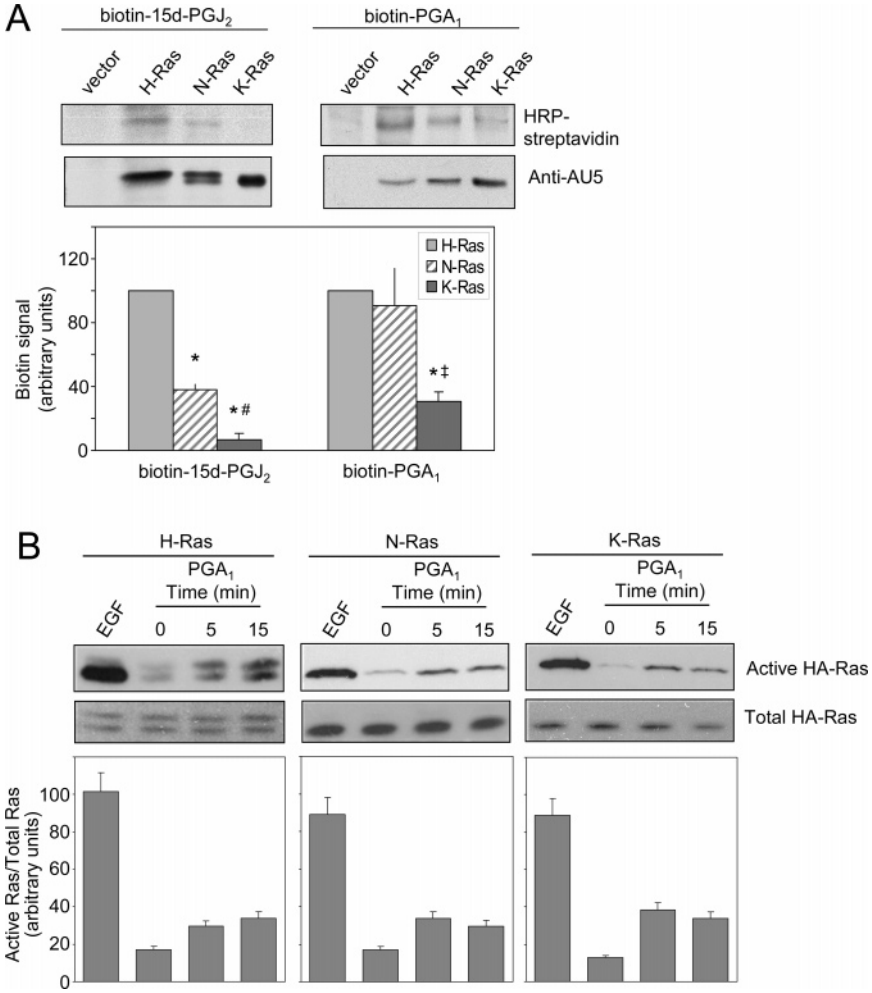

FIGURE 7: Modification and activation of H-, N-, and K-Ras proteins by different cyP. (A) COS7 cells were transfected with plasmids coding for H-Ras, N-Ras, or K-Ras4B, as indicated, and incubated with biotinylated cyP. Incorporation of biotin-15d-PGJ_2 and biotin-PGA_1 was assessed and quantified as described in the legend for Figure 5. The results shown are average values ± SEM of three experiments processed in duplicate (*$p < 0.05$ vs H-Ras for every biotinylated cyP; #$p < 0.05$ vs N-Ras-biotin-15d-PGJ_2; ‡$p < 0.05$ vs K-Ras-biotin-15d-PGJ_2 by *t*-test). (B) Cells transiently transfected with the indicated plasmids were serum-starved for 18 h and then incubated with vehicle (−), 100 nM EGF for 10 min, or 10 μM PGA_1 for the indicated time periods. Levels of active Ras were assessed as in Figure 6B. The results shown are from a representative experiment. Similar results were obtained in three additional, separate experiments. The histograms show average values from four separate assays ± SEM.

role (*29*, *51*). In this regard, we have observed that treatment with 15d-PGJ_2 or PGA_1 does not increase the amount of H-Ras in P100 fractions from cells. However, it leads to an improved extractability in Triton X-100 (Gayarre et al., unpublished observations), which has been previously interpreted as a redistribution of H-Ras into disordered plasma membrane microdomains (*52*). Further work is needed to elucidate whether this effect may be a cause or a consequence of Ras activation. Conversely, the modification of cysteine 118 could exert an effect on GDP or GTP binding. In preliminary assays, however, we have observed that co-transfection with a dominant negative form of SOS1 reduced H-Ras activation by cyP (unpublished data). This argues against a direct effect of cyP on nucleotide exchange and suggests that activation of H-Ras by cyP requires the participation of exchange factors. Finally, the increase in active Ras observed could be the result of a complex balance among the potential modifications of Ras sulfydryl groups, as has been proposed for oxidative modifications (*36*, *44*). Other possibilities such as the potential interference of cyP addition with Ras protein−protein interactions should not be discarded.

In conclusion, the results reported herein open the way to study whether the differential selectivity of cyP interaction with Ras proteins can be exploited to modulate Ras-mediated processes. Besides, the finding that Ras proteins can be the site for nonenzymatic modification of cysteine residues by various electrophilic lipid moieties offers novel perspectives on the study of the complex mechanisms that control Ras targeting and signaling under conditions of oxidative stress.

ACKNOWLEDGMENT

We thank Beatriz Garzón for valuable help and M. Jesús Carrasco for technical assistance.

SUPPORTING INFORMATION AVAILABLE

Peptide fingerprint of control H-Ras. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI602389P