Expression−Activity Profiles of Cells Transfected with Prostaglandin Endoperoxide H Synthase Measured by Quantitative Fluorescence Microscopy†

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ABSTRACT: Transfection of *cos*-1 cells with either prostaglandin endoperoxide H synthase-1 (PGHS-1) or -2 (PGHS-2) results in a mixed population of cells containing a diverse range of expressed enzyme. The use of fluorescent substrates and antibodies, in conjunction with fluorescence microscopy, provides the means to quantitate expression and activity of the enzyme within individual cells. Data obtained from individual cells can be utilized to construct enzyme activity curves for a population of transfected cells. This method has been employed to prepare expression−activity profiles within a population of *cos*-1 cells expressing PGHS-1 or -2. A direct correlation was observed between enzyme expression and activity as measured in single cells. The data demonstrate that activity−expression analyses can now be performed within single adherent cells growing in tissue culture.

Prostaglandins are key mediators of inflammation (Masferrer *et al.*, 1994; Meade *et al.*, 1993; Mitchell *et al.*, 1993). Prostaglandin endoperoxide H_2 is synthesized by two related prostaglandin endoperoxide synthases (PGHS-1 and PGHS-2)[1] (Hemler *et al.*, 1976; Kujubu *et al.*, 1991). The two genes coding for these proteins have been mapped and found to be homologous, except for the first intron (Smith, 1992). Other differences between these two homologous proteins are as follows: (a) PGHS-1 is constitutively expressed in cells, while the expression of PGHS-2 appears to be regulated by growth factors, cytokines, and tumor promoters (Jones *et al.*, 1993; Kujubu *et al.*, 1991); (b) PGHS-2 is concentrated in the nuclear envelope, while PGHS-1 is a resident lumenal protein of the endoplasmic reticulum (Morita *et al.*, 1994; Otto & Smith, 1994). To pursue questions related to the structure, function, and localization of these enzymes, we transfected *cos*-1 cells with plasmids containing the protein coding regions of either native or mutagenized versions of PGHS-1 or PGHS-2. Immunofluorescent analysis of these enzymes within individual transfected cells demonstrated that their levels of expression varied considerably between cells. These results illuminate a significant problem associated with the use of bulk evaluations of enzyme activities in transfected cell lines. The variable expression of proteins in these cells and the possibility of an unusual pattern of cellular compartmentalization for overexpressed proteins (Wozniak & Blobel, 1992) can lead to inaccurate evaluations of enzyme activity. In such situations, the abnormal localization could conceivably lead to large amounts of an active enzyme residing in a subcellular compartment containing neither substrates nor cofactors.

In this paper, we describe the use of quantitative fluorescence microscopy to perform sequential measurements of enzyme activity and protein expression within individual transfected cells. Previously employed techniques used to measure enzyme activity within cells or tissues have not provided information concerning cell-to-cell variability for PGH synthase activity resulting from altered levels of expression or different intracellular locations of the enzyme. Such differences in topology or expression of PGH synthase within cell populations could have physiological significance for activity and regulation. These studies, performed in a large number of individual cells transfected with either PGHS-1 or PGHS-2, show that a direct correlation exists between enzyme expression and activity.

EXPERIMENTAL PROCEDURES

Materials. Dulbecco's modified Eagle medium (DMEM) was obtained from Life Technologies, Inc. Fetal calf serum and calf serum were purchased from Hyclone. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG, FITC-conjugated phalloidin, and flurbiprofen were purchased from Sigma (St. Louis, MO). Arachidonic acid was obtained from Cayman Chemical Co. (Ann Arbor, MI). 5- (and 6)-Carboxy-2′,7′-dichlorodihydrofluorescein diacetate (CDCF-DA) and 3,3′-dihexyloxacarbocyanine [$DiOC_6(3)$] were obtained from Molecular Probes (Eugene, OR). All other reagents were from common commercial sources.

Transient Expression of Proteins in cos-1 Cells. Cos-1 cells were cultured in Lab-Tek coverslip chambers (Nunc). Preparation of expression plasmids encoding the ovine S530N PGHS-1 mutant, which expresses peroxidase activity but not cyclooxygenase activity, and the ovine H386A PGHS-1 mutant, which expresses cyclooxygenase activity but not peroxidase activity, and for the human PGHS-1 and PGHS-2 have been described previously (Laneuville *et al.*, 1994; Shimokawa & Smith, 1991, 1992). Native and mutant ovine and human PGHS-1 and human PGHS-2 proteins were expressed following transfection of these plasmids into *cos*-1

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[1] Abbreviations: PGHS, prostaglandin endoperoxide synthase; CDCFDA, 5- (and 6-)carboxy-2′,7′-dichlorodihydrofluorescein diacetate; $DiOC_6$, 3,3′-dihexyloxacarbocyanine; PBS, phosphate buffered saline; DMEM, Dulbecco's modified Eagle medium; COX, cyclooxygenase; POX, peroxidase.

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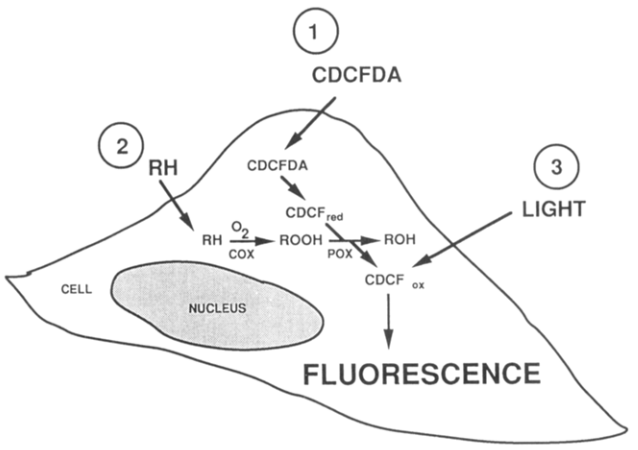

FIGURE 1: Histofluorescence assay for PGHS-1 activity. A description containing the details of the methodology is presented in the text.

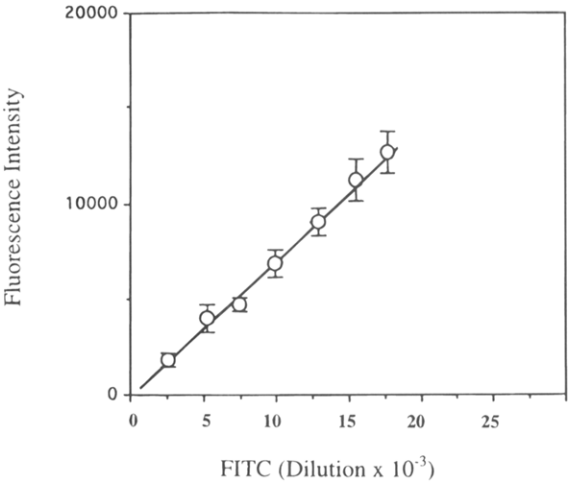

FIGURE 2: Determination of a linear range for quantitative fluorescence measurements. Fluorescence intensities were measured as a function of fluorophore concentration. Samples of FITC-labeled goat anti-rabbit IgG were prepared and analyzed as described in Experimental Procedures. Each point is the average of 20 measurements in different regions of the sample. The bars indicate the standard deviations from the mean.

cells as reported previously (Laneuville *et al.*, 1994; Otto *et al.*, 1993).

PGHS Isozyme-Specific Anti-Peptide Antibodies. Antibodies reactive with both ovine and human PGHS-1 were prepared against the peptide sequence Leu-Met-His-Tyr-Pro-Arg-Gly-Ile-Pro-Pro-Gln (residues 272−283 of ovine PGHS-1), which includes the trypsin cleavage site unique to ovine PGHS-1 (Otto & Smith, 1994). An antibody reactive with human PGHS-2 was prepared against the peptide sequence Ser-His-Ser-Arg-Leu-Asp-Ile-Asn-Pro-Thr-Val-Leu-Ile-Lys (residues 584−598 of murine PGHS-2) (Otto *et al.*, 1993; Otto & Smith, 1994). Peptide-specific antibodies were purified on peptide affinity columns (Otto & Smith, 1994).

Measurement of PGHS Activity by Histofluorescent Staining. Transfected *cos*-1 cells were washed with phosphate-buffered saline (PBS) containing both 0.1 mg/mL calcium and magnesium chloride (PBS+) and then incubated with CDCFDA (5 μM). Following uptake, the CDCFDA is converted to CDCF by endogenous esterases within the cells (Figure 1). Following the addition of arachidonic acid, the fluorescence intensities within cells were measured by fluorescence microscopy.

PGHS Immunocytochemistry. Following the measurement of PGHS activities, the *cos*-1 cells were washed in PBS, fixed for 10 min in 2% formaldehyde in PBS, and washed with 10% calf serum in PBS. CDCF is washed away during fixation. Affinity-purified anti-peptide antibodies to either PGHS-1 or −2 were added to the fixed cells in PBS/0.2% saponin/10% calf serum, and the samples were incubated for 30 min (Otto & Smith, 1994; Regier *et al.*, 1993). After washing in PBS containing 10% calf serum, the samples were incubated for 30 min with a 1:40 dilution of FITC-conjugated goat anti-rabbit IgG in PBS/0.2% saponin/10% calf serum. The samples were then washed with PBS/10% calf serum and rinsed with PBS. Controls included cells treated without primary antibodies and cells treated with a primary antibody in the presence of its cognate peptide (10 μM). The same cells that were examined for PGHS activity were selected for quantitation of bound fluorescence antibody.

$DiOC_6(3)$ Staining of Endoplasmic Reticulum. Samples previously examined for PGHS activity were treated with 1 μg/mL $DiOC_6(3)$ (Teraski & Reese, 1992) for 10 s at room temperature and rinsed with PBS. This procedure was performed while maintaining the cells on the microscope stage. CDCF fluorescence was not observed at the detector settings required to visualize the $DiOC_6(3)$.

F-actin Staining with FITC−Phalloidin. Following measurements of PGHS activities and fixation as described above, cells were incubated with a 1:40 dilution of FITC−phalloidin (Maruo *et al.*, 1992) for 30 min in PBS/0.2% saponin/10% calf serum. The samples were then washed with PBS/10% calf serum and then rinsed with PBS.

Fluorescence Microscopy. An Insight bilateral laser scanning confocal fluorescence microscope (Meridian Instruments, Okemos, MI) with an argon ion laser beam for excitation was used for fluorescent measurements of PGHS activities and expression. A 10× objective lens and a laser power setting of 30 mW was used to measure PGHS activity, antibody staining, and FITC−phalloidin labeling. A power setting of 1 mW was utilized for measurements with $DiOC_6(3)$.

RESULTS

The goal of these studies was to determine the relationship between immunocytofluorescent staining for PGHS protein and immunohistofluorescence staining for PGHS activity within single cells. *cos*-1 cells transfected with plasmids encoding PGHS-1 and PGHS-2 were utilized for these measurements. First, a concentration range was determined for fluorescein labeling that demonstrated a linear relationship between fluorescein fluorescence intensity and concentration (Figure 2). This range was determined for goat anti-rabbit IgG-conjugated fluorescein isothiocyanate (FITC goat anti-rabbit IgG). The FITC conjugate was diluted, and aliquots of the standard solutions were placed in Lab-Tek coverslip chambers and imaged with an Insight bilateral laser scanning confocal microscope.

These latter measurements provided a linear range for two types of fluorescence determinations: isozyme-specific antibodies were used to quantitate PGHS-1 or PGHS-2 proteins in single cells, and PGHS-1 and PGHS-2 activities were quantified using a histofluorescence staining method. Briefly, the peroxidase substrate, 5- (and 6)-carboxy-2′,7′-dichlorodihydrofluorescein (CDCF), was passively incorporated into cells as the acetoxymethyl ester, 5- (and 6)-

carboxy-2′,7′-dichlorodihydrofluorescein diacetate (CDCFDA) (Bass *et al.*, 1983; Cathcart *et al.*, 1986) (Figure 1). Following partitioning into the cell, cytoplasmic esterases cleave the methyl esters and trap the resultant charged CDCF. CDCF is a common cosubstrate for peroxidases (Cathcart *et al.*, 1986) and, accordingly, will serve as a reducing substrate for the peroxidase activities of PGHS-1 and PGHS-2. PGG_2 generated by the cyclooxygenase activity of PGHS enzymes upon the addition of arachidonate is reduced to PGH_2 by the peroxidase activities of the PGHS enzymes, with resultant oxidation of CDCF to a fluorescent product that can be detected by fluorescence microscopy. In all measurements to be described, the enzyme activity and the enzyme concentration within each cell were analyzed sequentially. Only cells demonstrating fluorescence intensities within the range of linear response shown in Figure 2 were utilized for single cell expression−activity analysis.

A typical series of experiments is presented for *cos*-1 cells transfected with a plasmid encoding ovine PGHS-1 (Figure 3).[2] Cells in Figure 3a show a broad range of activities, as measured by CDCF fluorescence. A cell-by-cell analysis shows that these same cells have a similarly diverse pattern of enzyme expression (FITC−anti-rabbit) (Figure 3b) that is correlated within each cell with the previous activity measurements (Figure 3a). No similar correlation is observed between the same cell labeled for activity (Figure 3c) and 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$], which partitions into all intracellular membranes (Figure 3d). Nontransfected cells show neither arachidonate-stimulated fluorescence (Figure 3e) nor anti-PGHS-1 staining (Figure 3f). As expected, there was $DiOC_6(3)$ staining (Figure 3h) in the nontransfected cells but no PGHS activity (Figure 3g).

The quantitation software of the Insight bilateral laser scanning confocal microscope was used to prepare histograms comparing activity and expression, as determined by fluorescence intensity (Figure 4a,b). A similar comparison was also made for cells transfected with a cDNA encoding human PGHS-2 (Figure 4c,d). Figure 4, panels a,b and c,d, demonstrate that, for large populations of cells, there is a relationship between the intensity of antibody staining (PGHS expression) and fluorescence from oxidized CDCF (enzyme activity), respectively.

The utility of this approach for examining expression−activity relationships for mutant proteins is shown in Figure 5. Cells were transfected with native ovine PGHS-1 and either a plasmid encoding the S530N PGHS-1 mutant, which has peroxidase but not cyclooxygenase activity, or a plasmid containing the H386A PGHS-1 mutant, which has cyclooxygenase but not peroxidase activity. The levels of expression for mutant and native proteins in the transfected cell lines were similar (8−12%). However, because CDCF oxidation requires coexpression of both cyclooxygenase and peroxidase activities, only the native enzyme is active, as determined by histofluorescence. This result shows that the modifications that lead to enzyme inactivation do not result in alterations in the level of protein expression. Interestingly, cotransfection of *cos*-1 cells with both the S530N and H386A PGHS-1 mutants leads to coexpression within some cells and a concomitant low level of activity that presumably reflects metabolic cooperation between the two inactive species (i.e., the cyclooxygenase activity of the H386A

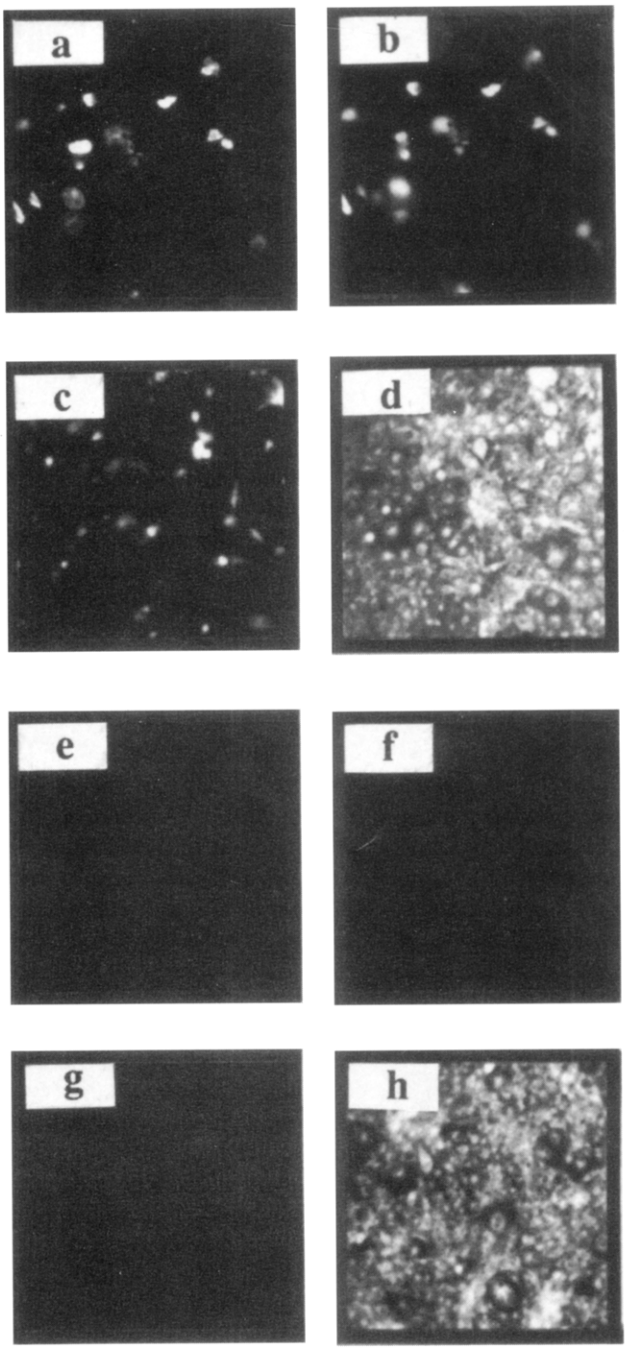

FIGURE 3: Expression−activity profile for ovine PGHS-1 in transfected *cos*-1 cells. *cos*-1 cells were transfected with a pSVT7 expression vector containing the coding region of ovine PGHS-1 (a−d) or subjected to a sham transfection (e−h). In two cases (d and h), cells were stained using $DiOC_6(3)$. Activity and expression of PGHS-1 in transfected *cos*-1 cells were sequentially measured by histofluorescence and immunofluorescence, respectively, on single cells as described in Experimental Procedures. Digital fluorescence images of the distribution of fluorescence intensity for the following: (a) *cos*-1 cells transfected with PGHS-1 and then stained for PGHS-1 activity; (b) the same cells as in panel stained for PGHS-1 protein; (c) *cos*-1 cells transfected with PGHS-1 and then stained for PGHS-1 activity; (d) the same cells as in panel c stained with $DiOC_6(3)$; (e) sham-transfected *cos*-1 cells stained for PGHS-1 activity; (f) the same cells as in panel e stained for PGHS-1 protein; (g) sham-transfected *cos*-1 cells stained for PGHS-1 activity; and (h) the same cells as in panel stained with $DiOC_6(3)$. Images are ×200.

[2] Addition of flurbiprofen (10^{-4} M), a cyclooxygenase inhibitor, 1 min prior to the addition of arachidonate completely blocked histofluorescence staining, indicating that the fluorescence resulted from cyclooxygenase activity.

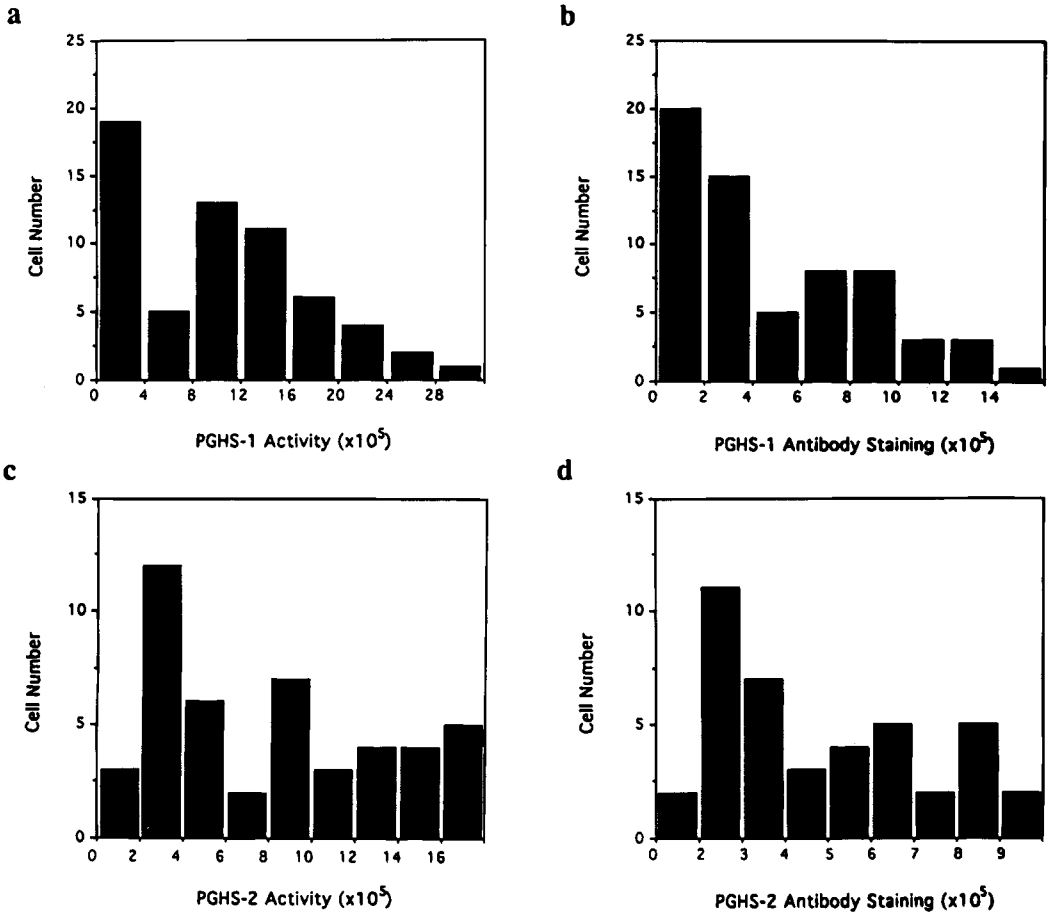

FIGURE 4: Cell population comparisons of PGHS-1 and PGHS-2 activities and expression. *cos*-1 cells were transfected with pSVT7 containing the coding region of ovine PGHS-1 (a and b) or pOSML containing the coding region of human PGHS-2 (c and d), and the intensities of histofluorescence staining (a and c) and immunofluorescence staining (b and d) were then determined as described in the text. The ordinate represents the number of cells, while the abscissa is an arbitrary scale of fluorescence intensity.

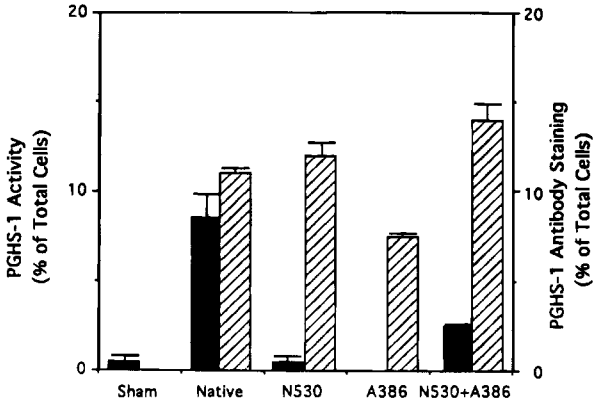

FIGURE 5: Expression—activity profiles for native and mutant forms of ovine PGHS-1. *cos*-1 cells were sham-transfected or transfected with pSVT7 expression vectors containing the coding regions for native ovine PGHS-1; S530N PGHS-1 (N530); H386A PGHS-1 (A386); or both S530N and H386A PGHS-1 mutants (N530 + A386). The cells were then stained sequentially for enzyme activity by histofluorescence (solid box) and for enzyme protein using immunocytofluorescence (hatched box). In each experiment, a total of 200 cells was counted.

mutant enzyme complementing the peroxidase activity of the S530N mutant enzyme).

In Figure 6, scatter plots are presented to show the correlation between activity and either level of expressed PGHS or amount of F-actin or total endomembrane. As shown for both PGHS-1 (Figure 6a) and PGHS-2 (Figure 6b), a good correlation exists between expression and activity. No correlation was observed between F-actin staining/PGHS-2 activity (Figure 6c) or $DiOC_6(3)$ staining/PGHS-2 activity (Figure 6d) in cells transfected with a cDNA encoding human PGHS-2. These results mirror those obtained with cells expressing PGHS-1 (data not shown).

DISCUSSION

Fluorescent probes have traditionally provided the sensitivity and chemical flexibility to serve as reporter substrates and antibody labels for measuring cellular enzyme activities and protein concentrations (Loew, 1993; Simon *et al.*, 1994; Taylor & Wang, 1978). The use of flow cytometry (Lynch *et al.*, 1991) provides a tool to perform multiple fluorescence measurements capable of quantitating either protein activities or protein concentrations within populations of single cells. Such studies, however, are limited by (a) an inability to quantitatively demonstrate relationships between ionic or enzymatic activities and the expression of the requisite enzymes or channels within the same cell and (b) the inability to examine the topology of activity—expression relationships within a cell. Indeed, the importance of providing a detailed cellular map of enzyme activity, expression, and subcellular localization within single cells is highlighted by our recent observation that, unlike PGHS-1, PGHS-2 is localized not only to the endoplasmic reticulum but also to the nuclear envelope (Morita *et al.*, 1995). These differences in localization may have significant implications for enzyme function, accessibility to substrate, and intracellular signaling.

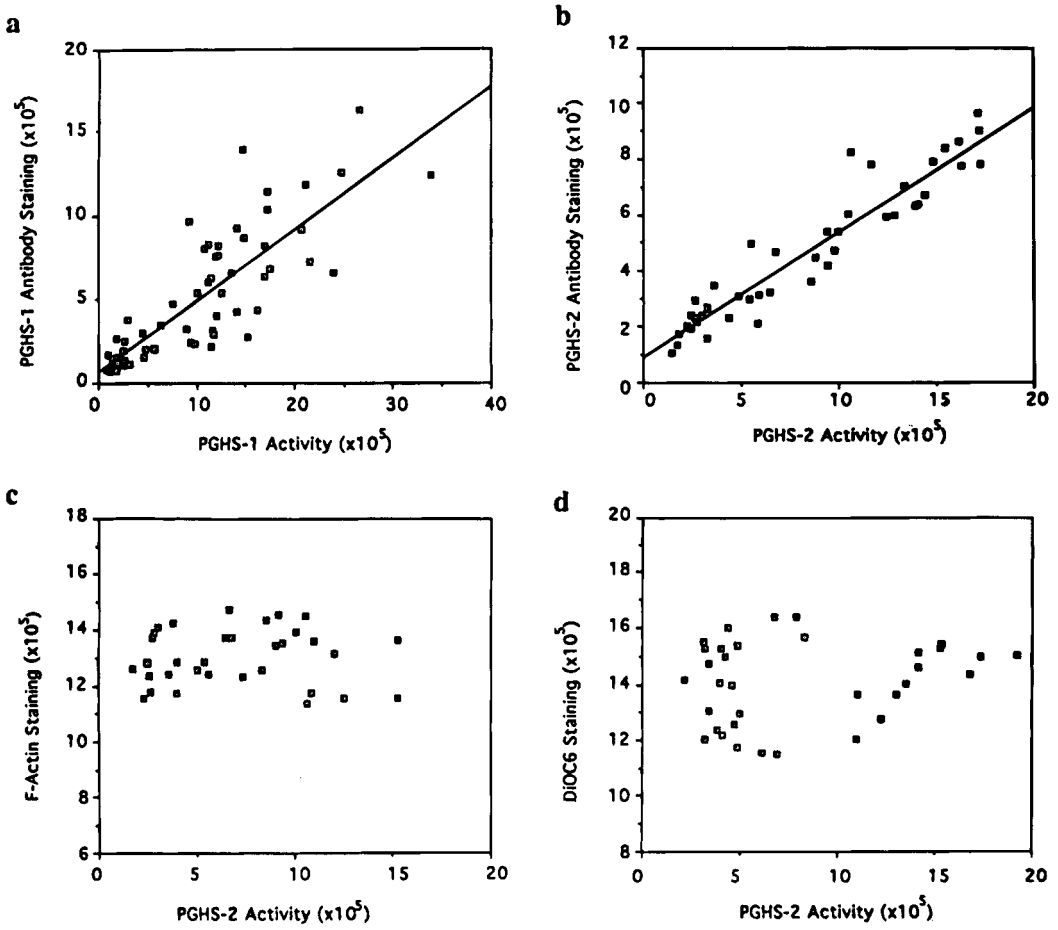

FIGURE 6: Scatter plots demonstrating the correlation between expression and activity for both PGHS-1 or PGHS-2 in single cells. *cos*-1 cells were transfected to express either ovine PGHS-1 (a) or human PGHS-2 (b–d) as described in the legend to Figure 4. The intensities of histofluorescence staining for enzyme activity and immunofluorescence staining for enzyme protein were then determined in individual cells as described in the text. Following this analysis, the cells were stained with FITC–phalloidin (F-actin stain) (c) or $DiOC_6(3)$ (d). Correlation coefficients for panels a and b are 0.72 and 0.95, respectively.

The present studies demonstrate that the analytical limitations of flow cytometry can now be overcome utilizing quantitative fluorescence microscopy. Since the use of conventional fluorescence microscopy for high-resolution quantitative measurements within cells is prone to significant signal-to-noise attenuation that results from out-of-focus light, confocal imaging will be an important requirement for future use of these *in situ* methods. The capability of the confocal microscope to provide both defined axial and lateral resolution offers the means to impose a "virtual cuvette" on a fluorescent sample (Lynch *et al.*, 1991; Wade *et al.*, 1993). The defined volume of fluorescence emission obtained from such a virtual cuvette can be used to establish quantitative comparisons of fluorescence intensities within and between cells.

The present paper provides validation for the use of quantitative fluorescence microscopy to sequentially determine both enzyme activity and concentration within single cells in tissue culture. Enzyme expression–activity profiles are demonstrated for *cos*-1 cells transfected with native and mutant PGHS-1 and PGHS-2. These enzymes exist in an active form in cells but form PGH_2 only when arachidonate is supplied exogenously or mobilized from endogenous lipids in response to hormonal stimulation (Smith, 1992). Our results indicate that the observed activity of both PGHS-1 and PGHS-2 is proportional to the amount of PGHS protein expressed when activity and expression are measured at the level of the single cell. These measurements now provide support for a technique that utilizes transfected cell lines to prepare activity curves for proteins in their native environments. Similar experiments may now be envisioned using fluorescence confocal microscopy and multiple fluorescent probes with distinguishable fluorescence emissions to perform expression–activity measurements on a number of proteins within the same cell.

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