A novel method for observing proteins *in vivo* using a small fluorescent label and multiphoton imaging

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A novel method for the fluorescence detection of proteins in cells is described in the present study. Proteins are labelled by the selective biosynthetic incorporation of 5-hydroxytryptophan and the label is detected via selective two-photon excitation of the hydroxyindole and detection of its fluorescence emission at 340 nm. The method is demonstrated

in this paper with images of a labelled protein in yeast cells.

Key words: fluorescence, green fluorescent protein (GFP), 5-hydroxytryptophan, imaging, labelled protein, phosphoglycerate kinase.

INTRODUCTION

Studying proteins in intact cells using fluorescence microscopy techniques is an area of intense interest in cell biology. Proteins are usually detected following the selective attachment of a fluorescent chromophore, accomplished by chemical modification of the purified protein *in vitro* and microinjection into cells or by genetic fusion with GFP (green fluorescent protein) to create fluorescent chimaeras *in situ* [1,2]. One approach to the potential problem created by the very large size of the GFP label, and variants of GFP (25–27 kDa), was developed by Tsien and co-workers [3]. They genetically incorporated into the target protein a short α -helix containing four cysteine residues, with a very high affinity ($K_d \sim 10^{-11}$ M) for a relatively small, membrane-permeable, fluorescein derivative administered extracellularly. This label adds much less mass than GFP and offers much greater versatility in terms of the sites and type of label attachment. Elegant though this technique is, the helix and fluorescent chromophore still add in excess of 1 kDa to the labelled protein and there may be problems with toxicity [4]. A related approach uses a fusion between the target protein and the DNA repair enzyme, AGT (O6-alkylguanine-DNA alkyltransferase), which can be alkylated *in vivo* at a specific cysteine residue by fluorescent analogues of its substrate. However, the label, which is the enzyme + fluorescent derivative, is very large and the experiment may need to be performed in AGT-deficient cell lines to avoid labelling of endogenous AGT [5].

Our solution to the problem of label size was to biosynthetically introduce the label as a modified amino acid. We had shown previously that 5-fluorotryptophan could be incorporated selectively into yeast proteins by inducing their synthesis in the presence of the labelled tryptophan [6–9]. ¹⁹F-NMR detection of these labelled proteins could then be used to report on their ligand binding properties and mobilities *in vivo*. These NMR spectra also showed that labelling was relatively specific, although some label was incorporated into other cell proteins. We show in the present study that a protein can be labelled in a similar way with 5OHTrp

(5-hydroxytryptophan) and that fluorescence can be detected from this labelled protein in an intact cell using multiphoton imaging.

EXPERIMENTAL

Methods

Protein labelling

PGK (phosphoglycerate kinase) was labelled by inducing its expression in the presence of 5OHTrp, using a galactose-inducible expression vector [8]. Briefly, 2×10^8 cells were used to inoculate a 50 ml culture containing 2% (w/v) glucose, 2% (w/v) bactopectone and 1% yeast extract. When the cells were in stationary phase, 5 ml of a 25% (w/v) galactose solution was added to the media. After 2 h, 2.5 ml of a 0.2% solution of L-5OHTrp was added and the culture was incubated for a further 24 h before cell harvesting. The increase in enzyme concentration, following galactose induction, was assessed using a spectrophotometric assay of the enzyme's activity in cell lysates, as described in [8]. For microscopy experiments, the cells were placed on a 1% agarose gel, which prevented cell movement.

Multiphoton microscopy

The two-photon microscopy apparatus was constructed in the Central Laser Facility of the Rutherford Appleton Laboratory using a modified Bio-Rad MRC500 confocal scanning system. Laser light at a wavelength of 628 ± 2 nm was obtained from an optical parametric oscillator (APE, Coherent, Santa Clara, CA, U.S.A.) pumped by a titanium-sapphire, 81 MHz, mode-locked laser (Spectra-Physics, Darmstadt, Germany), with a pulse width of 120 fs. The light was focused to a diffraction-limited spot through an air UV objective ($\times 40$, NA 0.85; Olympus, Tokyo, Japan) and specimens were illuminated at the microscope stage (modified Olympus IMT-2 with UV transmitting optics) by passing the beam through the MRC500 scan head. Fluorescence emission was passed through a 340 ± 15 nm interference filter

Abbreviations used: AGT, O6-alkylguanine-DNA alkyltransferase; GFP, green fluorescent protein; EGFP, enhanced GFP; 5OHTrp, 5-hydroxytryptophan; PGK, phosphoglycerate kinase.

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(U340, Comar Instruments, Cambridge, U.K.). The scan was operated in the normal mode, and line, frame and pixel clock signals were generated and synchronized with an external fast microchannel plate photomultiplier tube, which was used as the detector. These were linked via a time-correlated single-photon counting PC module SPC700 (Becker and Hickl, Berlin, Germany).

Image analysis

Images (6 bit, 256×256 pixels) were exported from Becker and Hickl software as bitmaps and converted into TIFF files. Image analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). A thresholding function was used to remove background pixels lying outside the cells, and the means for the remaining pixels was determined.

RESULTS AND DISCUSSION

Tryptophan and its derivative, 5OHTryp, have similar absorption maxima at 250–280 nm. However, 5OHTryp also has a significant shoulder to this peak at 315 nm. Excitation of fluorescence at 315 nm, in a mixture of 5OHTryp-labelled and unlabelled proteins, produces a fluorescence signal at 338 nm that is almost exclusively from the 5OHTryp label [10]. Incorporation of this label has been shown, in many cases, to have little effect on the function of those proteins that have been studied [11]. We have reported previously that 5OHTryp-labelled proteins can be detected *in vivo*, based on the excitation of this shoulder to the absorption peak, by fluorimetry [12,13]. However, attempts to use single-photon confocal microscopy to detect 5OHTryp-labelled proteins were severely limited by the UV light required to excite fluorescence, since this produced high levels of background or autofluorescence that made detection of the label difficult and rendered the technique impractical as a method for detecting a protein *in vivo*. We show here that this problem can be overcome by using multiphoton excitation with photons of longer wavelength [14]. In this technique, two or three photons excite a fluorophore nearly simultaneously and sum their energies to simulate a single photon of one-half or one-third the wavelength respectively. This requires extremely high photon fluxes, which can be achieved at the focus of a microscope objective of high numerical aperture illuminated with a pulsed laser. As in conventional confocal microscopy, the image is acquired by scanning the focus point. However, with multiphoton imaging, excitation is restricted to the focal plane and thus out-of-focus autofluorescence, photodamage and light scattering are all reduced. Webb and co-workers used three-photon excitation to image serotonin distribution in live cells, demonstrating high levels of serotonin in secretory granules in rat basophilic leukaemia cells [15]. Serotonin, like 5OHTryp, is a 5-hydroxyindole. We have used two-photon excitation here to excite fluorescence selectively from a 5OHTryp-labelled protein in the yeast *Saccharomyces cerevisiae*.

PGK, a cytosolic glycolytic enzyme, was selectively labelled by inducing its synthesis in the presence of 5OHTryp. The yeast strain and galactose-inducible vectors used have been described previously [8]. Cells were imaged by two-photon excitation of the 5OHTryp chromophore, using an excitation wavelength of 628 nm, and the fluorescence emission was measured at 340 nm (Figure 1). Variation in the conditions of galactose induction resulted in the production of cells with 1.5-fold (Figure 1C) and 4.6-fold (Figure 1D) increases in PGK concentration, as determined from measurements of enzyme activity. Controls were prepared by inducing cells, containing an empty vector that lacked

Figure 1 Images of cells containing 5OHTryp-labelled PGK

(A) Cells transformed with an empty vector (pKV49), lacking the PGK coding sequence, and galactose-induced in the presence of unmodified tryptophan. Scale bar, $20 \mu\text{m}$. (B) Cells transformed with the empty vector and induced in the presence of 5OHTryp. (C) Cells transformed with a vector expressing PGK (pKV43) and induced, in the presence of 5OHTryp, to give a 1.5-fold overexpression of the enzyme. (D) Cells transformed with pKV43 and induced, in the presence of 5OHTryp, to give a 4.6-fold overexpression of the enzyme. (E, F) Magnified view of the cells shown in (B) and (D) respectively. The images were obtained by two-photon excitation at 628 nm and acquisition of the fluorescence at 340 nm. Each image is the sum of four scans. The highest signal intensity in these images corresponds to $32\,000$ photon counts $\cdot \text{pixel}^{-1} \cdot \text{s}^{-1}$.

Figure 2 Quantitative analysis of image intensities in images acquired from cells containing labelled PGK

Photon counts $\cdot \text{pixel}^{-1} \cdot \text{s}^{-1}$ are plotted against fold induction of the enzyme, where 0-fold induction represents images acquired from cells transformed with an empty vector and galactose-induced in the presence of unmodified tryptophan (see Figure 1A), and 1-fold induction represents images from the same cells but induced in the presence of 5OHTryp (Figure 1B). The remaining points show the mean pixel counts in cells induced in the presence of 5OHTryp and overexpressing PGK 1.5- and 4.6-fold respectively.

the PGK coding sequence, in the presence of either 5OHTryp (Figure 1B) or unmodified tryptophan (Figure 1A). Quantitative analysis of the intensities in these and other images acquired from these cells was performed and the results are shown in Figure 2.

The image intensities in cells transformed with the empty vector and induced in the presence of unmodified tryptophan represent the true background from other chromophores in the sample (Figure 1A and '0-fold induction' in Figure 2). The intensities in the same cells induced in the presence of 5OHTryp (Figure 1B and '1-fold induction' in Figure 2) represent background, including residual unincorporated 5OHTryp, plus misincorporated label, i.e. 5OHTryp incorporated into proteins other than PGK. Galactose induction of PGK expression, in the presence of 5OHTryp, resulted in a directly proportional increase in image intensity, showing that with a 4.6-fold induction of PGK, nearly 70 % of the signal must have come from the labelled protein. We have estimated previously that the PGK concentration in non-induced cells is approx. 50 μM [8], and therefore the PGK concentration in the fully induced cells is of the order of 230 μM , or 180 μM labelled protein assuming that the labelling efficiency is 100 %. If this is the case here, then the increase in image intensity of approx. 13 000 counts/s between 1- and 4.6-fold PGK induction would correspond to a labelled protein concentration of 180 μM (360 μM 5OHTryp since there are two tryptophan residues per 45 kDa monomer) or 3600 counts $\cdot\text{s}^{-1} \cdot (100 \mu\text{M } 5\text{OHTryp})^{-1}$. This is greater than that observed with the pure amino acid, where 100 μM 5OHTryp gave a background-corrected intensity of approx. 2000 counts under similar conditions, suggesting that the fluorescence is enhanced when the amino acid is incorporated into PGK. However, there are many uncertainties in this calculation, including the intracellular enzyme concentration and the efficiency of labelling. Photobleaching, which was observed with the pure amino acid (results not shown), may also influence these estimates. At excitation intensities similar to those used here (2–3 kW/cm²), tryptophan showed significant bleaching and this was greater with the pure amino acid than when the amino acid was incorporated into a protein [16].

The low probability of multiphoton excitation requires high photon flux densities. In these experiments, the average laser power at the sample was approx. 19 mW. This is lower than the power used by Maiti et al. [15] to image serotonin distribution in mammalian cells, although they used a longer wavelength (700 nm). At the power levels used here, there were no visible signs of cell damage, although we have not ruled out the possibility that there was a loss of cell viability. Since there is no simple relation between photodamage and energy density, this would need to be tested [17].

The sensitivity of labelled protein detection is 10–100 times lower than that observed with conventional dye molecules (see e.g. [5]); however, this disadvantage has to be offset against the potential advantages of using a much smaller label. This sensitivity limit will, of course, be reduced if the target protein contains several tryptophan residues. Incorporation of 5OHTryp at ligand-binding sites may allow measurements of ligand binding *in vivo*, through effects on 5OHTryp fluorescence emission and lifetime [18], and therefore possibly the development of novel protein-based probes for a variety of intracellular metabolites and ions [7]. In addition, fluorescence anisotropy measurements on proteins labelled with 5OHTryp, which is superior to tryptophan as an anisotropy probe [18], could be used to probe protein's rotational mobility in the cell.

We attempted to label two proteins, PTB1 [19] and Vg1 RBP [20], in mammalian cells by transiently transfecting mouse C2C12 cells with vectors expressing the EGFP (enhanced GFP)-tagged proteins under the control of a CMV (cytomegalovirus) promoter. The cells were subsequently placed in a serum-free medium for 5 h and then in a serum-containing medium plus 1 mM or 100 μM 5OHTryp. Examination of the EGFP fluorescence 24 h later showed that the cells had expressed relatively high levels

of the EGFP-tagged PTB1 and Vg1 RBP, which showed predominantly nuclear and cytoplasmic localizations respectively. However, two-photon excitation at 628 nm produced a level of fluorescence emission at 340 nm that was only slightly greater than that observed in cells that had been incubated with tryptophan instead of 5OHTryp and which showed no evidence of significant localization (results not shown). This result is consistent with a lack of incorporation of 5OHTryp into proteins in mammalian cells [21] and suggests that the low level of fluorescence observed was due to unincorporated label and background fluorescence. Detectable levels of 5OHTryp may be achievable however by prior transfection of the cells with an orthogonal tryptophanyl-tRNA synthetase-mutant opal suppressor tRNA^{Trp} pair [21], which allows the efficient and selective incorporation of 5OHTryp into mammalian proteins in response to the codon TGA and allows mutagenesis of the tryptophan codons in PTB1 and Vg1 RBP to TGA. A possibly simpler alternative would be to micro-inject recombinant proteins that had been labelled in yeast or *Escherichia coli*. The latter approach would have the added advantage of reducing the background due to unincorporated label.

In conclusion, two-photon excitation of 5OHTryp-labelled proteins provides a novel method for investigating the properties of proteins *in vivo*, which may be useful in those situations where the large size of conventional fluorescent labels, particularly GFP and its variants, precludes their use. The method has been demonstrated in yeast, although, in principle, it should also be applicable to mammalian cells.

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