Protein Labeling

Methotrexate Conjugates: A Molecular In Vivo Protein Tag**

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Characterization of the distribution, fate, and intracellular chemical environment of proteins inside living cells is critical to the study of cell biology. Toward this end, green fluorescent protein and its variants (GFPs) have been used most prominently as markers or tags to monitor the localization and fate of proteins in vivo. In this application, a gene that encodes a GFP is fused to the gene that encodes a protein of interest. The resulting gene fusion is expressed, and the cells are examined by fluorescent microscopy.[1,2] A drawback to GFPs, however, is that their spectral and structural characteristics are interdependent.[3] Whereas mutagenesis has led to the development of differently colored GFPs, including cyan, green, yellow, and blue variants, and a red-emitting protein dubbed DsRed has been cloned from *Discosoma*,[4–6] it has been difficult to engineer GFP variants with well-resolved absorption and emission spectra for multicolor co-localization and fluorescent resonance energy transfer (FRET) applications, and in particular to obtain a well-behaved red variant. A protein-bound fluorophore that has its fluorescent properties uncoupled from its peptide sequence would allow greater freedom in the design of in vivo protein labels.

We address the limitations of GFPs with a method to specifically target cell-permeable small-molecule flurophores to selected proteins in vivo. The method leverages the noncovalent interaction between *Escherischia coli* dihydrofolate reductase (DHFR) and fluorescently labeled methotrexate (Mtx-F*; Figure 1). To demonstrate this method, we expressed DHFR-fusion proteins localized to either the plasma membrane or the nucleus in Chinese hamster ovary (CHO) cells and added TexasRed–methotrexate (Mtx–TR) to the growth media. The Mtx–TR bound noncovalently to DHFR and effectively labeled the localized fusion proteins.

The DHFR–Mtx protein-labeling method improves upon previously reported strategies that rely on covalent or non-

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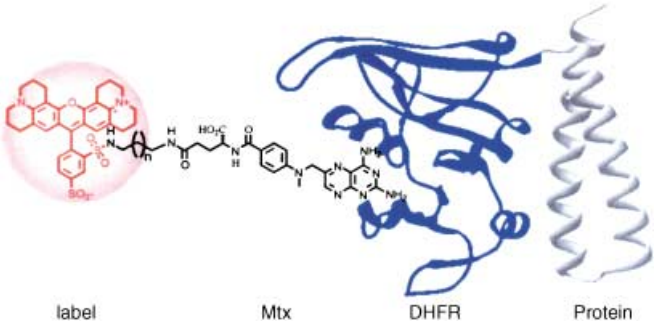

Figure 1. Schematic representation of the DHFR–Mtx protein-tagging system. A Mtx-conjugated fluorophore or other label (shown here, TexasRed) noncovalently binds DHFR, which is expressed in vivo as a fusion to the tagged protein of interest.

covalent labeling of fusion proteins with fluorescent ligands.[7–13] Farinas and Verkman labeled a single-chain antibody fusion with a fluorescein-conjugated hapten and optically measured the pH value of Golgi bodies in vivo.[7] A similar strategy to measure the pH values of organelles by using a biotin–avidin interaction was reported by Wu et al.[8] However, neither antibodies nor avidin make good receptors for general intracellular protein labeling. Farinas and Verkman reported that their antibody did not express well in cellular reducing environments, and avidin expresses as a 63-kDa tetramer. Fluorescent biarsenical ligands (FlAsH) target genetically encoded tetracysteine motifs (Cys-Cys-X-X-Cys-Cys, where X is any amino acid except cysteine).[9,10] It has been found, however, that the FlAsH derivatives bind nonspecifically to endogenous, cysteine-rich proteins.[11] More recently, a modular system based on the covalent labeling of O^6-alkylguanine-DNA alkyltransferase (hAGT) fusion proteins with fluorescently labeled O^6-benzylguanine derivatives (BG) was reported by Keppler et al.[12]

We chose DHFR and Mtx-F* as our protein–ligand label because DHFR and Mtx bind one another with particularly high affinity and because the interaction between the two has been extensively characterized both biochemically and structurally.[14–16] Mtx binds DHFR with subnanomolar affinity,[15] and the rate constant (k_{off}) for the dissociation of Mtx bound to the *E. coli* DHFR in a ternary complex with NADPH has been measured ($10^{-4}\ s^{-1}$).[16] Based on this high binding affinity and favorable kinetics, it is expected that Mtx analogues should stoichiometrically label DHFR fusions and, though noncovalent, the DHFR–Mtx complex should have a reasonable half-life. Furthermore, Mtx has been shown to inhibit the proteolytic degradation of DHFR.[17] Mtx can be chemically modified without disrupting receptor binding by adding modifications at the γ-carboxylate position.[14,15] Thus, it should be possible to chemically link Mtx to a wide variety of fluorophores. Indeed, a number of methotrexate-conjugated fluorophores are commercially available (Molecular Probes, Eugene, OR). Fluorescein methotrexate has been used as a probe for the overexpression of DHFR in methotrexate-resistant cell lines,[18,19] and as a fluorescent probe in protein-complementation assays.[20,21] DHFR is a monomeric, 18-kDa protein (by comparison, GFP is a 27-kDa protein), and functional DHFR fusion proteins have been used in a variety of biochemical applications, including a yeast three-hybrid screen for protein–small molecule interactions.[19–22] Given these characteristics, it should be possible to express DHFR as a fusion to native proteins with little likelihood of interference with native protein function. Despite the commercial availability of fluorescent methotrexate analogues, and the well-characterized stoichiometric labeling of intracellular DHFR with fluorescein methotrexate, the DHFR–Mtx interaction has not yet been exploited as a generic system for in vivo protein labeling.

To demonstrate the utility of DHFR–Mtx-F* as a protein label, we used fluorescent microscopy to observe the labeling of *E. coli* DHFR fusion proteins in mammalian cells with TexasRed-conjugated Mtx (Mtx–TR). To avoid potential background fluorescence or toxicity as a result of binding of Mtx–TR to endogenous DHFR, we used a DHFR-deficient CHO cell line in our studies.[23] We targeted the label to the plasma membrane (PM) in CHO cells by expressing DHFR with an N-terminal fusion of the 10-amino acid myristoylation/palmitoylation sequence from the protein lyn. This signal sequence (MGCIKSKGKD) fused to the N-terminus confers PM localization.[24] DHFR was also targeted to the nucleus by encoding three copies of the nuclear localization signal (NLS) of the simian virus 40 large T-antigen (DPKKKRKV) at its N-terminus.[25] We expected to obtain microscopic images of our modified CHO cells that revealed characteristic PM or nuclear fluorescence upon labeling with Mtx–TR.

To label PM-targeted DHFR in CHO cells, we modified a mammalian expression vector, pECFP-N1-lyn, that had the lyn PM-targeting sequence appended N-terminally to cyan fluorescent protein (CFP).[24] We used standard molecular biology techniques[26,27] to replace the DNA that encodes the CFP in pECFP-N1-lyn with DNA that encodes the *E. coli* DHFR, yielding pLM1208. Replacement of the PM-targeting sequence DNA with DNA that encodes three copies of the NLS yielded plasmid pLM1264. We transiently transfected DHFR-deficient CHO cells grown on 22-mm^2 coverslips with pLM1208 or pLM1264. Approximately 24 h after transfection, growth media that contained Mtx–TR (2 μM) was added to the cells. After approximately 20 h of incubation with Mtx–TR, the cells were washed twice with PBS, mounted in media without Mtx–TR, and imaged using a scanning confocal microscope.

Fluorescent microscopy of CHO cells transfected with pECFP-N1-lyn that encodes the CFP control verified PM targeting due to the N-terminal signal sequence (Figure 2a). Microscopic images of CHO cells transfected with pLM1208 that encode the DHFR tag and labeled with Mtx–TR revealed a similar pattern of illumination consistent with PM fluorescence (Figure 2b). Cells labeled with Mtx–TR initially showed a high background fluorescence that diminished as unbound Mtx–TR leaked from the cells into the media. After about 20 min, distinct PM labeling with low background fluorescence was visible. The red fluorescence of the PM-targeted DHFR–Mtx–TR complex was clearly visible up to at least 1 h after removal of the small molecule. Microscopy of cells transfected with pLM1264 showed a distinct nuclear fluorescence (Figure 2c). Control experi-

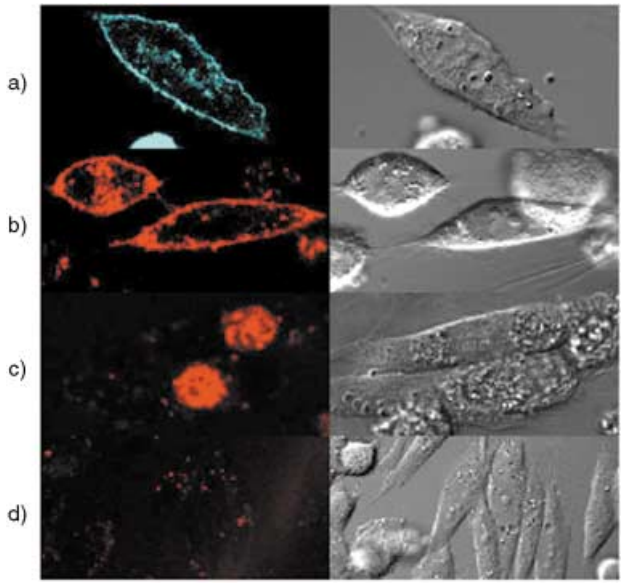

Figure 2. Noncovalent labeling of localized *E. coli* DHFR in DHFR-deficient CHO cells. Confocal micrographs show fluorescence in left column, differential image contrast (DIC) images in right column: a) PM fluorescence in cells transiently expressing cyan fluorescent protein fused to PM-targeting sequence (excitation: 457 nm). b) DHFR-deficient CHO cells transiently expressing PM-targeted DHFR. Cells were incubated in media containing Mtx–TR (2 μM) for 20 h, washed with PBS, mounted in media without Mtx–TR, and imaged. c) Cells expressing nucleus-targeted DHFR, treated as in b). d) CHO cells transiently expressing PM-targeted DHFR that were incubated in media containing Mtx–TR (2 μM) and Mtx (10 μM). Excess Mtx binds available DHFR, preventing PM labeling with Mtx–TR. For images b–d, excitation was carried out at 565 nm.

ments established that the red fluorescent signal was dependent on the specific labeling of PM-targeted or nucleus-targeted DHFR with Mtx–TR. Incubation of CHO cells transiently expressing PM-targeted DHFR with media containing a fivefold excess of Mtx relative to Mtx–TR resulted in no PM labeling (Figure 2d). Similarly treated cells expressing nucleus-targeted DHFR as well as cells that were not transfected and incubated in media containing Mtx–Tr (2 μM) showed no localized fluorescence (data not shown). These results suggest that Mtx outcompetes Mtx–TR for binding to eDHFR, further indicating that the red fluorescent signal we observed was due to Mtx–TR binding to the DHFR portion of the fusion protein. Results similar to those shown in Figure 2 were observed when fluorescein-conjugated Mtx was used to label localized DHFR (data not shown).

We next sought to determine whether Mtx–TR could be used to label *E. coli* DHFR fusion proteins in non-DHFR-deficient mammalian cells, despite the possibility of toxicity or background fluorescence as a result of binding of Mtx–TR to endogenous DHFR. To demonstrate labeling in wild-type (DHFR +/+) cells, we transfected NIH 3T3 fibroblasts with pLM1208 (encoding PM-targeted *E. coli* DHFR) and prepared them for microscopy under similar conditions as for DHFR −/− CHO cells. To mitigate any potential toxic effects of Mtx–TR, we supplemented the growth media with thymidine (30 μM). We imaged the cells using a microscope capable of either epifluorescent illumination or total internal reflection (TIRF) illumination. Epifluorescence (Figure 3a) cannot distinguish between Mtx–TR bound to endogenous DHFR in the cytosol and Mtx–TR bound to PM-localized

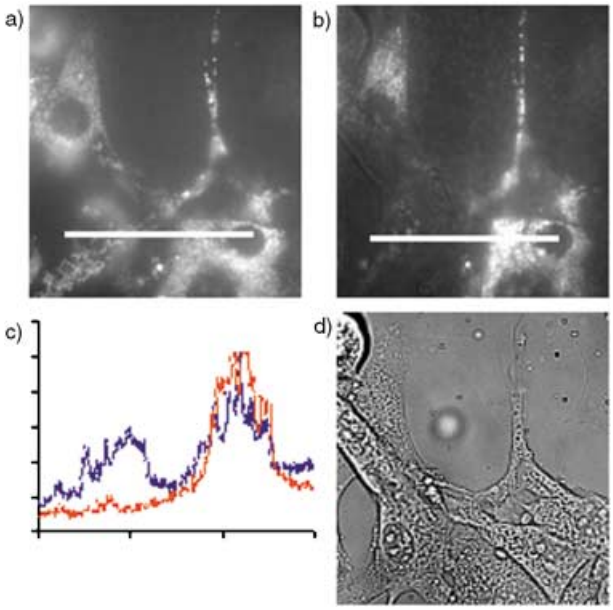

Figure 3. TIRF microscopy eliminates background fluorescence resulting from Mtx–TR binding to endogenous, cytosolic DHFR in wild-type cells. NIH 3T3 fibroblast cells were transfected with DNA encoding PM-targeted *E. coli* DHFR. a) Epifluorescence micrograph reveals fluorescence of Mtx–TR bound to native, cytosolic DHFR and/or PM-localized *E. coli* DHFR. b) TIRF micrograph of the same cells shows only fluorescence of Mtx–TR bound to PM-localized *E. coli* DHFR in cells expressing the fusion protein (on the right-hand side of the image). Background fluorescence in cells not expressing PM-targeted DHFR is effectively excluded. c) Plot of fluorescence intensity (arbitrary units) vs. pixel value along the white line shown in images a) and b). The blue line is epifluorescence intensity (image a), and the red line is the value of TIRF fluorescence intensity (image b). d) DIC micrographs of the cells.

E. coli DHFR. However, when using TIRF illumination, it was possible to selectively excite and view the Mtx–TR-labeled, PM-localized *E. coli* DHFR (Figure 3b). In TIRF illumination mode, an evanescent wave is used to excite fluorophores within approximately 100–200 nm of the coverslip surface.[28] This mode effectively limits excitation to fluorophores localized to the basal plasma membrane of cells grown on coverslips. These results show firstly, that wild-type cells tolerate exposure to Mtx–TR, and secondly, that the Mtx–DHFR method is viable for the labeling and detection of PM-localized proteins by microscopic methods that optically eliminate background interference as a result of binding of Mtx conjugates to endogenous DHFR. Using RNA interference methods to knock out native DHFR expression,[29] it should be possible to label and detect *E. coli* DHFR fusion proteins localized to any subcellular compartment in wild-type mammalian cells.

The commercial availability of TexasRed–methotrexate makes the DHFR–Mtx protein-labeling method immediately

useful to cell biologists as an alternative to the problematic DsRed fluorescent protein. The real strength of this approach, however, is that the chemical functionality is uncoupled from the labeling mechanism. Therefore, fluorescent conjugates other than TexasRed could be employed, as well as other tags such as photoaffinity labels, NMR-active nuclei, or PET tags. Currently, we are engineering Mtx analogues that bind to DHFR variants, but not wild-type DHFR. This should allow labeling in non-DHFR-deficient cell lines and differential labeling of several proteins in the same cell. This approach is well-precedented in the development of modified chemical dimerizers of FKBP (FK506-binding protein).[30] Given the ease with which fluorescent Mtx analogues can be prepared and the efficient site-specific labeling of a DHFR fusion protein described herein, we anticipate that DHFR–Mtx-F* complexes will find broad use as protein labels in biochemical and cell biological applications.

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