Covalent labelling of fusion proteins in live cells *via* an engineered receptor–ligand pair†

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An engineered, orthogonal ligand receptor pair has been exploited as a method to covalently label fusion proteins with small molecule probes in live cells.

Proteins are routinely expressed in cells with an additional polypeptide label. Labels can be used to evaluate protein distribution, movement, interactions, and local chemical environment.[1] Introduction of labels can also facilitate protein purification and the patterning of proteins into microarrays.[2] In response to drawbacks of genetically encoded protein tags, various techniques have been developed to enable the specific tagging of proteins with synthetic small molecules.[3] Particular effort has been devoted towards labelling proteins with small molecule fluorophores. The labelling of proteins as fusions to green fluorescent protein (GFP) family members has been remarkably useful for the study of protein function, yet suffers from a number of shortcomings.[1] The spectral properties of these proteins are limited. Only a few pairs of proteins are suitable for fluorescence resonance energy transfer (FRET). Improved properties, such as well-resolved absorption and emission spectra, have proven difficult to engineer. Small molecule fluorophores have a number of advantages in these criteria. Additionally, small molecule probes can be used for a variety of other techniques, including affinity purification, photocrosslinking, luminescence, magnetic resonance imaging, optical coherence tomography, and near-infrared fluorescence.[3,4]

This paper describes a method for the site-specific, covalent labelling of fusion proteins with small molecules by exploiting an engineered receptor–ligand pair. Our lab has previously reported a selective cyclophilin A–cyclosporin A (CypA–CsA) receptor–ligand system created *via* exo-mechanism proximity accelerated reactions.[5] The approach involves the introduction of a mildly electrophilic group on the ligand and a suitably positioned nucleophilic thiol (*via* an engineered cysteine mutation) on the receptor to achieve selectivity through the formation of covalent complexes. Engineered covalent complexes have also been used to obtain allele-specific modulators of protein function,[5] to screen for drug fragments on protein surfaces,[6] to create antibodies with infinite affinity,[7] to map receptor–ligand interfaces,[8] and for kinase-substrate crosslinking.[9] Here, we employ ligand-induced proximity accelerated reactions with the CypA–CsA system as a model for a new approach towards the covalent labelling of fusion proteins in live cells with small molecule probes.

This engineered CypA–CsA system has many attributes that are suitable for a technique to covalently label fusion proteins in live cells. The CypA protein tag is only 18 kDa and is monomeric.[10] Its chaperone activity as a *cis–trans* proline isomerase could be considered as relatively inert to the cell. CypA is tolerant to expression as a fusion protein at either the N or C terminus.[11] Also the creation of covalent complexes by exo-mechanism proximity accelerated reactions enables the selective labelling of target proteins even in the presence of endogenous receptor cyclophilins, and therefore does not require the use of knockout cell lines.

We prepared an active ester of cyclosporin A (CsA), 1, *via* olefin metathesis as described previously.[12] Using diethanolamine as a trifunctional linker allowed for subsequent appending of a 5/6-carboxytetramethylrhodamine (TMR) fluorophore and an acrylamide electrophile (Scheme 1). CsA analogues modified at the butenylmethylthreonine residue have shown to retain binding affinity to cyclophilins, yet lose their immunosuppresive activity.[13] Previously prepared CsA conjugates containing amide bond linkages were found to be cell impermeable (data not shown).

The reactivity of the electrophilic, fluorophore containing CsA derivative, **3**, was assessed with the CypA mutant P105C. This mutation near the CsA binding site has been shown in previous studies to be well suited for reactions with electrophilic CsA derivatives.[5] The compound demonstrated rapid, $t_{1/2} \approx 15$ min, alkylation of the protein *in vitro* as determined by gel shift on denaturing SDS-PAGE (Fig. 1). The small amount of unlabelled protein remaining after extended reaction time is likely due to the presence of inactive protein in the preparation.

To assess the specificity of labelling among other proteins in live cells, COS-7L cells (simian kidney cell line) were transiently transfected with a P105C–CypA expression vector and treated with CsA–EL–TMR at 1 μM (Fig. 2). The reactivity of the compound for the target protein was highly selective (Fig. 2B, lane 3) with only minimal background labelling. The labelled target protein comprised only a small portion of the total protein in the cell lysate: approximately 0.1% (wt/wt) as determined by comparing fluorescent band intensity to *in vitro* labelled P105C–CypA.

To further determine the suitability for selective labelling in biological systems, live HepG2 cells (human liver carcinoma cell line) expressing a GFP–P105C–CypA fusion protein were treated with CsA derivatives and imaged by fluorescence microscopy (Fig. 3). Incubation with CsA–EL–TMR, **3**, shows labelling with the fluorophore selectively in transfected cells, as indicated by the co-localization of green and red cells (Fig. 3A and Fig. 3C). As a control experiment to indicate that observed fluorescence was due

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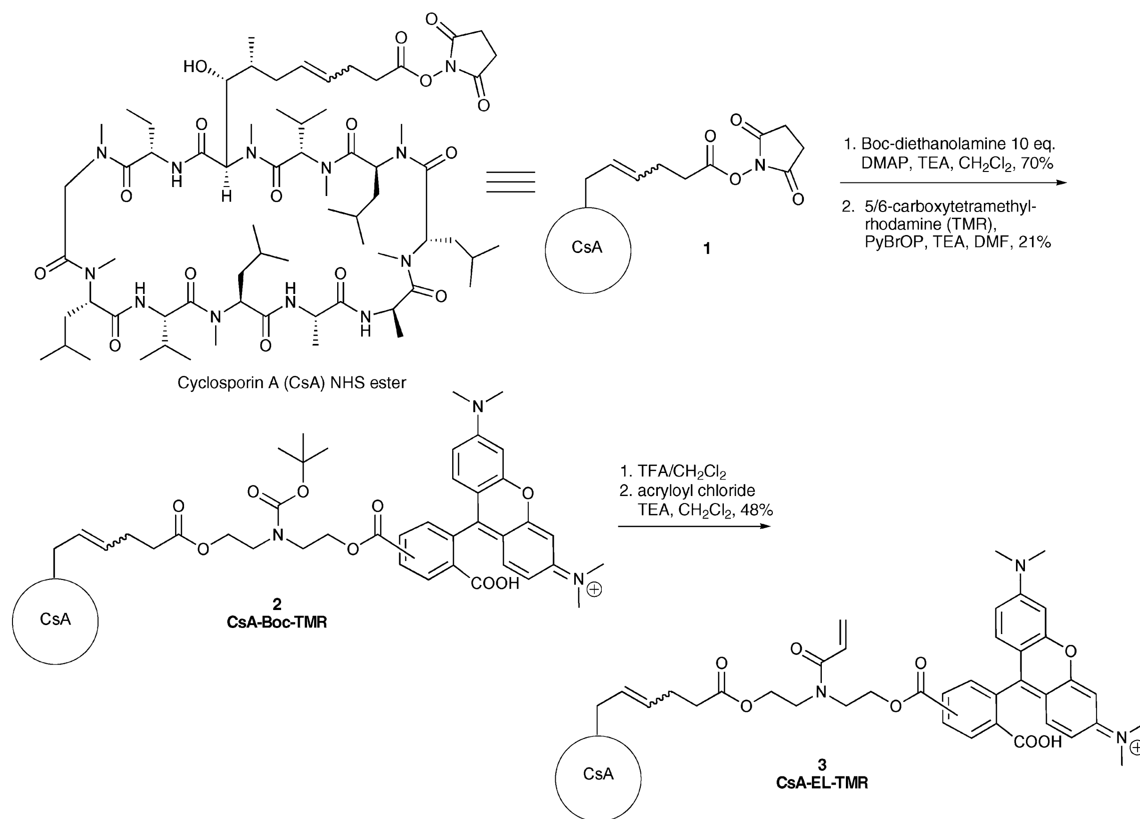

Scheme 1 Synthesis of a CsA derivative containing an electrophile and a flouorophore (CsA-EL-TMR).

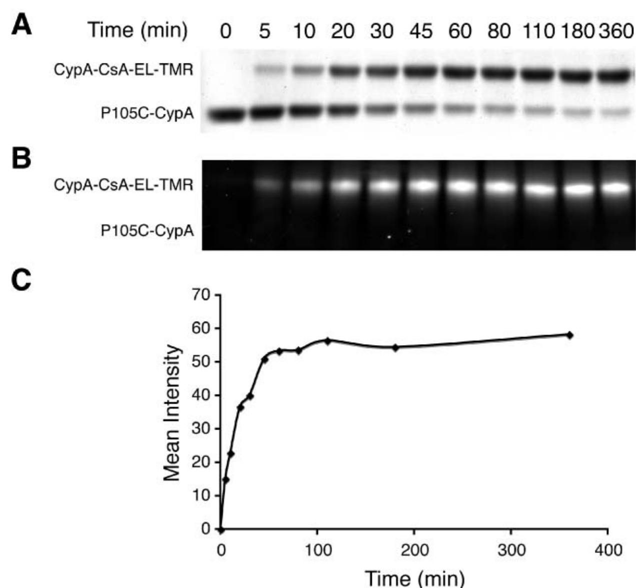

Fig. 1 SDS-PAGE analysis of labelling of CypA mutant P105C by CsA-EL-TMR, **3**. Reactions contained 5 µM protein and 10 µM **3** in 50 mM phosphate buffered saline pH 7.5 with 0.05% Tween-20 and 1 mM reduced glutathione at 37 °C. (A) Image of a Coomassie blue stained gel. 1 µg protein per lane. (B) Fluorescence gel image. 15 ng protein per lane. (C) Plotting of product intensities obtained from gel in (A).

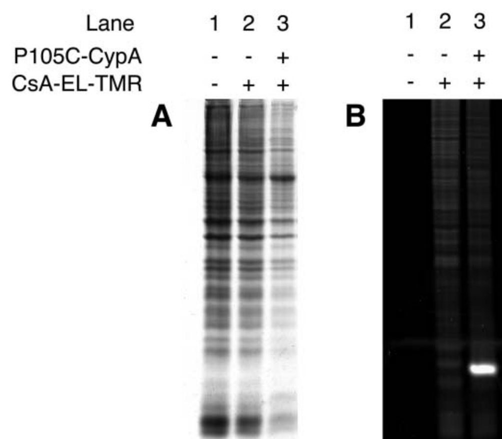

Fig. 2 SDS-PAGE analysis of P105C-CypA labelling in live cells. 10 hours after transfection, COS-7L cells were treated with CsA-EL-TMR at 1 µM, where indicated. After 12 hours of incubation, cells were washed and lysed with SDS sample loading buffer. (A) Image of a Coomassie blue stained gel. (B) Fluorescence gel image. Lanes 1–3, 5 µl per lane from 250 µl whole cell lysate from 10 cm^2 well plate.

to the covalent labelling of GFP-P105C-CypA, cells were similarly treated with non-electrophilic CsA-Boc-TMR, **2**. This treatment resulted in no labelling of cells with the red fluorophore (Fig. 3B).

We found extended incubation and wash times (~18 h total) were necessary to maximize the signal of the TMR fluorophore, which could be attributed to limited membrane permeability by CsA–EL–TMR. In addition, the presence of green and red fluorescence correlated well in cells treated with CsA–EL–TMR (Fig 3A, Fig 3C), yet occasionally differences were observed in the relative intensity of the two fluorescence signals. This may have been due to variances in the permeability among the cells.

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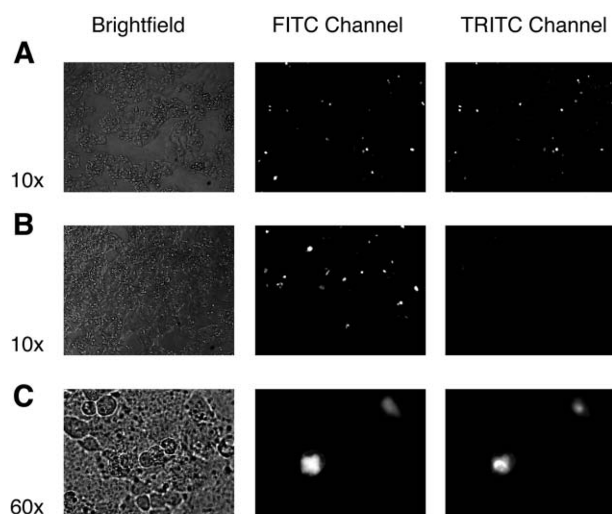

Fig. 3 Images of labelling of GFP-P105C-CypA fusion proteins in live cells. 24 hours post transfection, Hep-G2 cells were treated with 2.5 μM CsA and 2.5 μM CsA-EL-TMR (Panels A and C) or non-reactive CsA-Boc-TMR (Panel B). Cells were incubated for 18 hours and washed prior to imaging by fluorescence microscopy. FITC and TRITC channels represent green and red fluorescence, respectively. See ESI for colour version of Fig. 3†.

The use of a lipophilic fluorophore or a ligand–receptor pair with a more permeable ligand could ameliorate these problems. Minimizing the incubation time of cells with CsA–EL–TMR may decrease the amount of background labelling observed and also enable experiments which require labelling on a shorter timescale. Incubation times as short as five minutes have been reported with the fusion protein covalent labelling approach developed by Johnsson *et al.*, which demonstrated similar labelling kinetics *in vitro.*[14] Furthermore, the tight binding of the modified CsA derivatives for endogenous cyclophilin receptors (~5 nM K_d for CypA[15]) may contribute to difficulties in washing out unreacted CsA–EL–TMR from cells. Co-incubation with unmodified CsA, which likely binds with slightly higher affinity than CsA–EL–TMR, was used to accelerate wash out time. The use of receptor–ligand combinations with moderate affinity or without endogenous receptor proteins, such as engineered "bump-hole" pairs,[15] may address these issues.

Many approaches have been developed to label proteins in live cells with small molecule probes,[3] including approaches exploiting receptor–ligand interactions that enable non-covalent, reversible labelling of fusion proteins.[16] For many applications, however, covalent bond formation of a small molecule to a target protein is desirable. The technique outlined here represents an additional method for the covalent labelling of fusion proteins without the use of post-translational modifying enzymes or split inteins.[3] Similar techniques include the biarsenical fluorophore/tetracysteine motif system (FlAsH and ReAsH),[17] the benzylguanine derivative/O^6-alkyguanine-DNA alkyltransferase (AGT) system (SNAP-Tag),[12] and the aliphatic chloride derivative/dehalogenase system (HaloTag).[18] The increasingly modular nature of our approach offers some advantages (Fig. 4). Like the SNAP-Tag and HaloTag approaches, this method is general with respect to the identity of the label. Uniquely, the proposed technique could potentially be employed with any receptor–ligand pair. This could enable the rapid development of several new orthogonal labelling systems, which would not require rare, mechanism-based covalent inhibitors or extensive protein engineering.

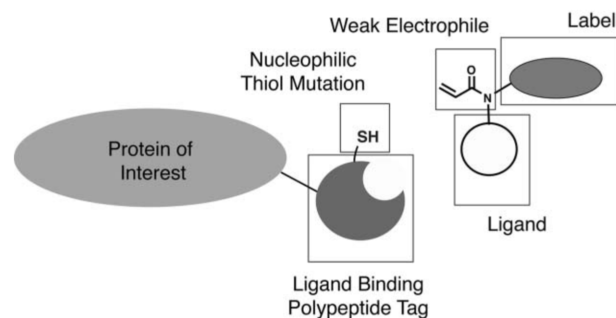

Fig. 4 Modules employed in this approach for the covalent labelling of fusion proteins.

Additionally, we envision this technique being applicable for receptor–ligand pairs with small peptide receptors such that the size of the genetically encoded tag can be minimized. Using phage display techniques, several short peptides have been identified, which have moderate affinity for small molecule ligands.[19,16d] In the creation of covalent complexes with engineered antibodies and acrylamide containing antigens, Meares *et al.*[6] observed efficient reactions even with weak binders ($K_d \approx 10^{-4}$ M).

In conclusion, we report a new approach for the covalent labelling of fusion proteins in live cells with small molecule probes, exploiting a proximity-accelerated reaction promoted by receptor–ligand binding. The technique offers promise for the development of additional tools for the study of protein function in biological systems.

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