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Site-Specific Epitope Tagging of G Protein-Coupled Receptors by Bioorthogonal Modification of a Genetically Encoded Unnatural Amino Acid

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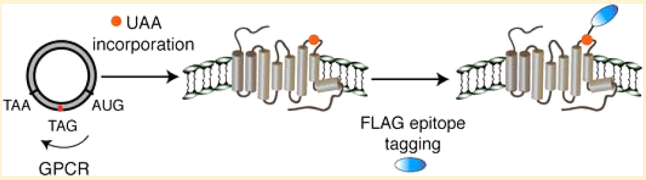
Supporting Information

ABSTRACT: We developed a general strategy for labeling expressed membrane proteins with a peptide epitope tag and detecting the tagged proteins in native cellular membranes. First, we genetically encoded the unnatural amino acid *p*-azido-L-phenylalanine (azF) at various specific sites in a G protein-coupled receptor (GPCR), C-C chemokine receptor 5 (CCR5). The reactive azido moiety facilitates Staudinger ligation to a triarylphosphine-conjugated FLAG peptide. We then developed a whole-cell-based enzyme-linked immunosorbent assay approach to detect the modified azF-CCR5 using anti-FLAG mAb. We optimized conditions to achieve labeling and detection of low-abundance GPCRs in live cells. We also performed an accessibility screen to identify azF positions on CCR5 amenable to labeling. Finally, we demonstrate a preparative strategy for obtaining pure bioorthogonally modified GPCRs suitable for single-molecule detection fluorescence experiments. This peptide epitope tagging strategy, which employs genetic encoding and bioorthogonal labeling of azF in live cells, should be useful for studying biogenesis of polytopic membrane proteins and GPCR signaling mechanisms.



The G protein-coupled receptors (GPCRs) are highly dynamic membrane proteins that activate and mediate numerous cellular signaling pathways. In the classical paradigm of GPCR signaling, receptor activation by an extracellular signal is accompanied by ligand-induced conformational changes.[1,2] Although recent advances have provided high-resolution crystal structures of several GPCRs, additional biophysical and biochemical techniques will be required for a precise understanding of the structural basis of receptor activation and allosteric mechanism. Our aim is to develop new approaches to site-specifically introduce minimally perturbing labels or probes into expressed receptors and prepare samples suitable for single-molecule fluorescence studies.[3] Conformational states of receptors under purified conditions[4,5] or in live cells[6,7] have been probed directly using fluorescence-based techniques. Chemistries that target cysteine thiol groups have been used to covalently attach fluorescent probes to GPCRs. However, this approach can be challenging because it requires the generation of cysteine-free background receptors to achieve single-site labeling. Such limitations can be overcome by site-specific introduction of unique functional groups that are not found in native proteins using unnatural amino acid (UAA) mutagenesis.[8]

A bioorthogonal chemical reporter strategy relies on the use of a biologically inert functional group, such as a ketone or azide that serves as a reactive chemical handle that can be covalently tagged using a selective chemical reaction. Ketones have been successfully incorporated into a variety of macromolecules to facilitate novel ligation strategies such as hydrazone reaction.[9−12] Azides can participate in bioorthogonal chemistries such as Staudinger ligation (Staudinger−Bertozzi ligation),[13,14] copper-free strain-promoted azide−alkyne cycloaddition (SpAAC),[15,16] and copper-catalyzed azide−alkyne cycloaddition (CuAAC).[17]

Amber codon suppression technology allows site-specific incorporation of UAAs with functional groups into an expressed target protein.[18−20] Previously, the Schultz group reported a series of engineered variants of *Escherichia coli* Tyr-tRNA synthetases (Tyr-RS) that recognize UAAs.[21] The combination of *E. coli* Tyr-RS and *Bacillus stearothermophilus* suppressor $tRNA^{Tyr}$ was used as an orthogonal pair for amber codon suppression in mammalian cells.[22] We built on these findings and optimized the methodology by generating a chimera of human and *B. stearothermophilus* $tRNA^{Tyr}$ to incorporate UAAs, such as *p*-benzoyl-L-phenylalanine (BzF), *p*-acetyl-L-phenylalanine (AcF), and *p*-azido-L-phenylalanine (azF), into low-abundance GPCRs heterologously expressed in mammalian cells.[23] The photolabile UAA, BzF, was exploited to map a ligand binding site on chemokine receptors, CXCR4 and CCR5.[24,25] The IR-active UAA, azF, was utilized to study conformational changes in the activation of rhodopsin using Fourier transform infrared (FTIR) difference spectroscopy.[26,27] In recent work, we compared two bioorthogonal labeling strategies using AcF or azF site-specifically introduced into the

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GPCR rhodopsin.[28] We concluded that the azido group served as a superior bioorthogonal handle, consistent with previous reports,[29,30] and we modified azF-rhodopsin with fluorescent probes using Staudinger ligation chemistry. A recent report of Staudinger ligations to two different azido amino acids showed that azF is only incompletely modified, attributed to the shorter backbone to azide linker length,[31] consistent with our observations.[28] Moreover, although the poor reaction kinetics and substoichiometric labeling properties of Staudinger ligation reagents can be overcome by the extraordinarily high efficiency of CuAAC, the toxicity of the Cu(I) catalyst makes it unsuitable for live cell labeling.[32] In contrast, although SpAAC reactions using cyclooctyne reagents are biocompatible similar to Staudinger ligation,[33,34] they result in background reactions with cysteine residues in proteins.[35]

The Staudinger ligation capitalizes on the specific reaction between a triarylphosphine and an azide to form an aza−ylide intermediate that undergoes cyclization followed by hydrolysis to form a stable amide-linked adduct. It has found useful applications in the labeling of recombinant proteins[13] and the modification of glycans on cell surfaces.[14] Phosphines have been derivatized with haptenes and epitope tags such as biotin[14] and FLAG peptide,[13] or with a variety of fluorescent probes to label and visualize cell surface glycoproteins in live cells.[36−41] The Staudinger ligation was employed to label residue-specific azido amino acids in proteins[13] or to site-specifically label azF-containing proteins in insect cells,[18] phage-display peptides, and recombinant proteins.[42]

We decided to develop a strategy for detecting modified receptors in live cells. Here, we modify an azF-GPCR with a FLAG peptide epitope tag by Staudinger ligation. We employed a cell-based ELISA strategy to detect low-abundance GPCRs labeled in a live cell context. The results of our ELISA demonstrate specific detection of azF receptors tagged with a FLAG peptide epitope. We exploit the semi-high-throughput property of our cell-based assay to identify sites on human CCR5 suitable for site-specific labeling. Finally, we focus on a preparative strategy for obtaining azF receptors that are bioorthogonally modified to lay the foundation for single-molecule fluorescence work.

MATERIALS AND METHODS

Materials. FLAG-triarylphosphine (FLAG-Phos), polyclonal anti-FLAG antibody produced in rabbit, and M2 mAb produced in mouse were obtained from Sigma. 1D4 mAb was obtained from the National Cell Culture Center; anti-CCR5 2D7 mAb was from BD Biosciences, and AcF and azF were purchased from RSP Amino Acids LLC and Chem-Impex International, respectively.

Plasmids and Site-Directed Mutagenesis. Plasmid pSVB.Yam carrying the gene encoding the amber suppressor tRNA was derived from *B. stearothermophilus* Tyr-$tRNA_{CUA}$ and described previously.[23] The aminoacyl-tRNA synthetases for AcF and azF were constructed as described previously. The C-terminal FLAG tag was removed from the aminoacyl-tRNA synthetase cDNAs by a deletion strategy using a Quikchange Lightning site-directed mutagenesis kit (Stratagene). The human CCR5 gene was in a pcDNA 3.1(+) plasmid and contained a C-terminal C9 epitope (TETSQVAPA) or 1D4 tag. The amber mutations were introduced into CCR5 using a Quikchange kit (Stratagene).

Heterologous Expression of Proteins with UAAs in Mammalian Cells. HEK 293T cells were maintained in DMEM-Q [4.5 g/L glucose and 2 mM glutamine (Gibco)] with 10% fetal bovine serum (Atlanta Biologicals) at 37 °C in 5% CO_2. Cells were transfected using Lipofectamine PLUS reagent (Invitrogen) with three vectors simultaneously containing the genes for the CCR5 amber mutant, suppressor tRNA, and aminoacyl-tRNA synthetase for AcF or azF. The ratio of DNA in micrograms was 1:1:0.1 (CCR5 amber:suppressor tRNA:UAA synthetase) prepared in DMEM. For transfection of wild-type (wt) CCR5, the microgram amount of DNA was maintained at a 1:10 ratio to the CCR5 amber mutants and supplemented with the appropriate amount of empty vector pcDNA 3.1(+). For mock-transfected cells, the amount of DNA corresponding to the amber receptor was substituted with empty vector pcDNA 3.1(+). For transfections in a 10 cm plate, 3.5 μg of amber receptor DNA was used with 10 μL of PLUS reagent and 17 μL of Lipofectamine. Transfections performed in a six-well plate were scaled to one-quarter quantity of all components; 3−4 h post-transfection, the medium was supplemented, yielding final concentrations of 10% fetal bovine serum with 0.5 mM UAA in DMEM-Q.

Bioorthogonal Peptide Epitope Tagging of CCR5 in Membranes. wt CCR5 or amber variant constructs were expressed by transient transfection in HEK 293T cells, and 48 h post-transfection, cells were harvested in 1× PBS supplemented with protease inhibitors aprotinin, leupeptin, and PMSF and pelleted at 3000*g* for 3.5 min using a tabletop centrifuge. Membranes were prepared as described previously from four 10 cm plates per receptor variant.[43,44] Receptors were labeled by resuspension of membranes from one plate in 100−125 μL of buffer A [20 mM Tris-HCl (pH 6.8), 150 mM NaCl, 1 mM $CaCl_2$, and 10 mM EDTA]. FLAG-Phos was added to a final concentration of 100 μM and the mixture incubated for 16 h at room temperature while being gently mixed on a rotatory wheel. Postlabeling membranes were washed three times with 3.5 mL of buffer A. Membranes were then solubilized in 1% (w/v) *n*-dodecyl β-D-maltoside (DM) (Anatrace) [50 mM Tris-HCl (pH 6.8) and 100 mM NaCl], and the CCR5 C9 C-terminally tagged receptor was immunoaffinity purified by overnight binding to 1D4-conjugated Sepharose resin at 4 °C. The resin was washed three times with wash buffer 1 (WB1) [0.1% DM and 50 mM Tris-HCl (pH 6.8)], followed by a single wash in wash buffer 2 (WB2) [0.1% DM and 0.1 M sodium phosphate buffer (pH 7.2)] in 30 min incubations at 4 °C. Receptor was eluted in elution buffer (WB2 with 0.18 mg/mL 1D5 peptide) and supplemented with 1× NuPAGE LDS sample buffer containing DTT.

Immunoblot Analysis. 1D4-purified receptor samples were subjected to SDS−PAGE (NuPAGE Novex 4 to 12% Bis-Tris gel) and then transferred to a PVDF membrane (Millipore) for immunoblotting. The PVDF membrane was blocked in 5% milk in 1× TBST for 1 h at room temperature. After being washed, the membranes were incubated with primary antibody in 0.5% BSA in 1× PBS on a shaker at 4 °C overnight. The blots were washed in 1× TBST and incubated with secondary antibodies in 5% milk in 1× TBST for 1 h. CCR5 expression was detected by blotting with the anti-1D4 mAb (1:2000 dilution) followed by HRP-conjugated anti-mouse IgG (1:15000 dilution). FLAG-Phos covalently tagged to receptor was detected with a polyclonal anti-FLAG antibody at a 1:3000 dilution followed by HRP-conjugated anti-rabbit IgG (1:20000 dilution). The membranes were incubated with

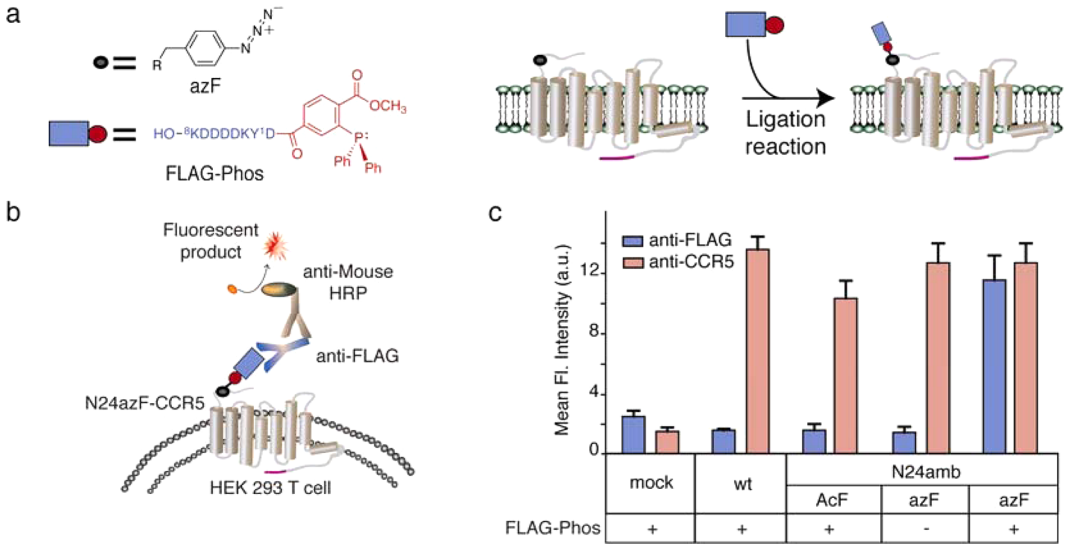

Figure 1. Cell-based immunoassay to detect azF-CCR5 site-specifically modified with FLAG-Phos. (a) Scheme showing the in-culture labeling of a GPCR genetically encoded with the UAA azF by Staudinger−Bertozzi ligation with a peptide epitope (DYKDDDDK, blue) conjugated to a triarylphosphine, FLAG-Phos (red). (b) Schematic diagram of the whole-cell-based ELISA used to monitor cell surface receptors modified with a peptide epitope tag. (c) Results of the ELISA of HEK 293T cells expressing CCR5, treated with 0.05 mM FLAG-Phos for 1 h under cell culturing conditions. Error bars represent the standard error of the mean of three or more replicate experiments. Anti-FLAG M2 antibody detection shows specific labeling of N24azF-CCR5 and the corresponding cell surface expression as detected by anti-CCR5 mAb 2D7.

chemiluminescent substrate (Thermo Scientific) and exposed to HyBlot CL autoradiography film (Denville Scientific, Inc.).

In-Culture Bioorthogonal Labeling of CCR5. wt CCR5 or amber variants were expressed in HEK 293T cells by transient transfection in six-well plates. Then 24 h post-transfection, cells were washed with 1 mL of 1× PBS. Cells were trypsinized with 200 μL of 0.25% trypsin (Invitrogen) per well for 3 min. Cells were supplemented with a 10-fold volume of DMEM-Q (10% FBS and 0.5 mM UAA) and gently resuspended for counting using a hemocytometer. Before the cells had been plated, the 96-well plate was treated with poly-D-lysine [0.01 mg/mL (Invitrogen)] for 15 min at room temperature followed by extensive washes with 1× PBS and air-dried. Cells were plated in 200 μL at a density of 60−80K cells/well of a pretreated 96-well plate [clear bottom, high binding EIA/RIA (Costar)]. Cells were adhered and ready for labeling 48 h post-transfection. Before being labeled, cells were washed three times with 100 μL each of 1× PBS [containing Ca^{2+} and Mg^{2+} (Invitrogen)] to remove any residual azido-containing medium. Labeling reagent was prepared from a 5−20 mM stock of FLAG-Phos (resuspended in 1× PBS or DMSO) to a final working concentration of 50−200 μM in 1× HBSS or PBS. Each well of the 96-well plate was treated with 60 μL of FLAG-Phos at the appropriate concentration and returned to 37 °C for the appropriate length of time (30 min to 4 h). No label-treated cells were maintained in the HBSS/PBS mixture for the specified duration. Postreaction labeling buffer was removed, and cells in the 96-well plate were further subjected to an ELISA.

ELISA Detection of the Labeled Receptor. All treatments during the ELISA were performed in blocking buffer (BB) [0.5% BSA in 1× PBS (Ca^{2+}/Mg^{2+})] unless otherwise indicated. Cells were washed two or three times with BB and then fixed with 100 μL of freshly prepared methanol-free paraformaldehyde per well for 20 min at room temperature. A 4% working stock was prepared from 16% paraformaldehyde (EMS) in 1× PBS (Ca^{2+}/Mg^{2+}). For wells intended for detection of the C-terminal 1D4 epitope tag or cytosolic proteins alone, cells were pretreated with 100% ice-cold methanol for 5 min on ice prior to primary antibody incubation. Following fixation, cells were washed three times with BB and incubated for an additional 15−30 min. Incubation with the primary antibody was conducted in 100 μL for 1.5 h on ice. The 2D7, anti-CCR5 conformation-sensitive antibody was originally used at a 1:200 dilution in BB and later optimized to a 1:500 dilution to detect cell surface-expressed CCR5. The anti-1D4 antibody was used to detect full-length receptor at a 1:2000 dilution in BB. Anti-FLAG antibodies (polyclonal or M2 mAb) were used at a 1:2000 dilution. Postprimary antibody incubation cells were washed three times with BB followed by incubation with a secondary antibody for 1 h at room temperature. Wells treated with the anti-FLAG polyclonal antibody were subjected to anti-rabbit HRP (1:2000 dilution), while all others were incubated with anti-mouse HRP (1:2000). After five careful washes with BB, cells were treated for 15 min with 50 μL of detection buffer: Amplex Red (Invitrogen), 20 mM H_2O_2, and 1× PBS in a 1:10:90 ratio. Spectral data were obtained at 530 nm (λ_{ex}) and 590 nm (λ_{em}) on a CytoFluor fluorescence plate reader instrument.

RESULTS

Cell-Based Assay for Detecting Epitope-Tagged azF-CCR5. We used CCR5 to demonstrate the feasibility of introducing a site-specific peptide epitope tag into an expressed GPCR. We used the bioorthogonal Staudinger−Bertozzi ligation chemistry to label receptors containing the UAA azF expressed on the surface of HEK 293T cells with the eight-residue FLAG peptide epitope conjugated to a triarylphosphine (FLAG-Phos) (Figure 1a). We designed a whole-cell-based ELISA strategy to achieve semi-high-throughput detection of modified receptors using a two-step antibody detection method (Figure 1b). First, anti-FLAG M2 mAb recognizes its specific epitope in FLAG-Phos-tagged receptors. Next, a secondary incubation with the anti-mouse IgG antibody conjugated to

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HRP serves to amplify the specific primary signal. The HRP oxidizes a fluorogenic substrate to yield a fluorescent product in the presence of hydrogen peroxide, which provides a quantitative measure of the detectable label. We employed a similar principle using the anti-CCR5 2D7 mAb to determine cell surface expression of the receptor.[45]

Staudinger ligation reagents tend to exhibit poor reaction kinetics and substoichiometric labeling.[31,32] Therefore, labeling low-abundance receptors such as GPCRs requires a multistep strategy. We employ here (i) site-specific introduction of the azF into CCR5, (ii) bioorthogonal labeling of the azF with an epitope tag, (iii) immunocomplex formation between the epitope tag and the specific mAb, (iv) secondary immunocomplex formation with the anti-mouse IgG, and (v) signal amplification with HRP. Additionally, the multistep strategy capitalizes on removal of unbound reagent at each step to capture labeling events in a live cell context. To further establish our procedure, we also considered factors such as the duration of labeling and optimal concentration of the FLAG-Phos reagent. Because we observed that amber mutant receptors containing azF typically express at a level that is ~20% of that of wt CCR5, we transfected cells expressing wt CCR5 with $^{1}/_{10}$ of the DNA dosage as compared with the amber mutant receptors to equalize expression levels and facilitate comparison.

ELISA results demonstrate the specific labeling of an azF-CCR5 variant at a solvent-exposed N-terminal position, N24azF, in culture with FLAG-Phos (Figure 1c). HEK 293T cells plated in a microtiter plate were treated with 50 μM FLAG-Phos under cell culturing conditions. Cells were washed of excess labeling reagent and subjected to whole-cell ELISA. The extents of labeling (blue columns) and cell surface-expressed receptor (red columns) were monitored side by side on cells from the same transfection in separate sets of wells to demonstrate the specificity of the antibodies for their respective epitopes. The anti-FLAG mAb displayed a specific signal only for the CCR5 amber mutant incorporated with the phosphine-reactive azido group treated with FLAG-Phos (N24amb/azF/+ condition). When azF was not provided to cells transfected with CCR5 N24amb and the amber suppression components for azF, receptor expression was not detected by the 2D7 mAb, concomitant with a lack of anti-FLAG detection (data not shown). Additionally, when cells expressing CCR5 N24azF were not treated with FLAG-Phos (N24amb/azF/− condition), a FLAG epitope-specific signal was absent. Therefore, removal of either reaction partner resulted in the loss of specific signal enhancement associated with FLAG peptide epitope tagging of CCR5. Little to no background label was detected in controls where CCR5 was not expressed. Minimal background was also observed when either wt CCR5 or CCR5 N24AcF, bearing an acetyl group that was chemically inert to triarylphosphine, was expressed.

Optimization of Reaction Conditions for Labeling azF-CCR5. We next analyzed the labeling reaction time course and FLAG-Phos concentration dependence with CCR5 N24azF in culture (Figure 2). Figure 2a shows data for 1 h labeling with increasing concentrations of FLAG-Phos (0−0.2 mM). A 3-fold enhancement in the ELISA signal was observed upon incubating cells with 200 μM versus 10 μM FLAG-Phos. Similarly, labeling with 50 μM FLAG-Phos for 4 h elicited a 2-fold enhancement in the signal as compared with labeling for 30 min (Figure 2b). Interestingly, labeling with 50 μM FLAG-Phos for 1 h resulted in a close to maximal signal. This confirms

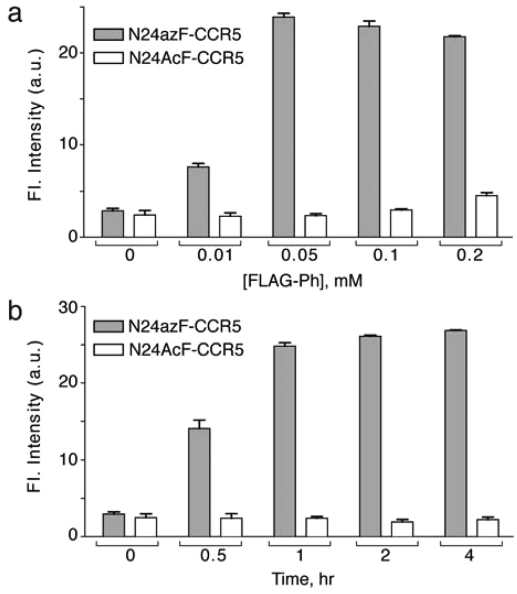

Figure 2. Optimization of the reaction conditions for labeling azF-CCR5 by Staudinger−Bertozzi ligation. (a) Concentration dependence and (b) time course analysis of specific labeling of N24azF-CCR5 with FLAG-Phos. HEK 293T cells expressing N24amb-CCR5 incorporated with AcF or azF were treated with 0−0.2 mM FLAG-Phos for 1 h (a) and with 0.05 mM FLAG-Phos for 0.5−4 h (b). The extent of label incorporated was detected by the anti-FLAG M2 antibody using the whole-cell-based ELISA. Error bars represent the standard error of the mean for triplicate measurements. Increasing the concentration and length of time of labeling resulted in a signal enhancement specific to the epitope tagging of N24azF-CCR5, while the AcF counterpart exhibited no label incorporation.

that conditions used for the experiments presented in Figure 1 are satisfactory. In all control experiments with CCR5 N24AcF treated similarly, nonspecific FLAG peptide epitope tagging was not observed, demonstrating the strict bioorthogonality of the Staudinger−Bertozzi ligation. Additionally, we highlight that all data depicted in panels a and b of Figure 2 were obtained in triplicate from a single experiment, demonstrating the potential of this detection method for use in semi-high-throughput screening.

Functional Characterization of Receptors Treated with FLAG-Phos. UAAs can be introduced into CCR5[23,25] and other GPCRs[24,26,27] without a loss of function. Here, we determined the agonist-dependent calcium mobilization of wt, AcF, and azF CCR5 variants after treatment with various FLAG-Phos concentrations (0−0.2 mM) (Figure 4 of the Supporting Information). With the exception of the nonfunctional variant R225azF, all other modified receptors exhibited a comparable functional response to a saturating concentration of the CCR5 agonist RANTES.

Accessibility Screen for Bioorthogonal Epitope Tagging of CCR5. We used CCR5 as a model system to perform an accessibility screen for bioorthogonal epitope tagging using Staudinger−Bertozzi ligation and the detection method described above. The semi-high-throughput property of the assay allowed us to evaluate the accessibility of several positions on CCR5 to FLAG-Phos. On the basis of the general topology of class A GPCRs, we selected positions on the extracellular, transmembrane, and cytoplasmic domains of the receptor. We determined the anti-CCR5 signal corresponding to the cell surface receptor expression of each variant (Figure 3a). Similar

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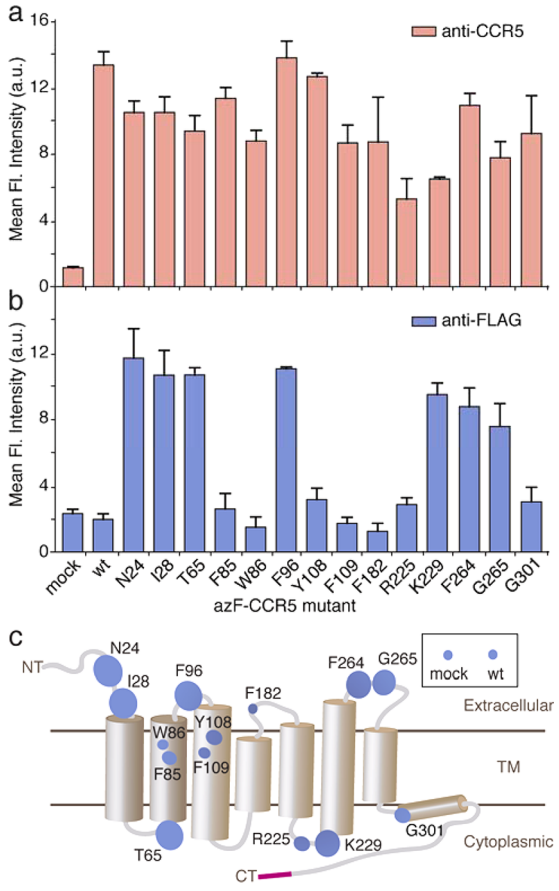

Figure 3. Accessibility screen for bioorthogonal modifications of UAAs in CCR5. (a) Cell surface expression of HEK 293T cells expressing azF-CCR5 variants and wt and mock-transfected controls probed with anti-CCR5 mAb 2D7 using the whole-cell-based ELISA. (b) Cells from the same transfection as panel a were treated with 0.05 mM FLAG-Phos for 1 h under cell culturing conditions. The extent of peptide epitope tagging was quantified by a subsequent ELISA using anti-FLAG M2 mAb. Error bars in panels a and b represent the standard error of the mean for two or more replicate data sets. (c) Relative extent of labeling indicated by the size of the diameter of the blue circles mapped onto a topological scheme of CCR5.

expression levels were observed across the series of positions genetically encoded with azF. HEK 239T cells expressing each azF-CCR5 variant were treated with 50 μM FLAG-Phos for 1 h under cell culturing conditions. The corresponding anti-FLAG signal for each azF-CCR5 variant was representative of the extent of FLAG peptide epitope tagging at that position (Figure 3b). The results are depicted on a topological scheme of CCR5 in which the size of the blue circles indicates the fold enhancement of the ELISA signal over the wt background (Figure 3c). Positions in the N-terminus and extracellular loops 1 and 3 show high levels of label incorporation, whereas positions buried in the transmembrane regions exhibit less labeling. Interestingly, positions on the cytoplasmic surface appeared to be labeled, a result contrary to our initial expectations because the fixative conditions utilized in the ELISA should preclude access of the antibody to the intercellular surface of the cell. Therefore, in the following sections, we describe control experiments in which this observation was investigated further.

Test for Membrane Permeability of FLAG-Phos. To assess the membrane permeability of FLAG-Phos, we utilized a cytosolic protein, firefly luciferase, containing azF at a solvent accessible position to demonstrate labeling in cells. After cells expressing the wt Fluc or azF-Fluc (Y70azF-Fluc) had been incubated with 0.5 mM FLAG-Phos for 1 h at 37 °C, cells were lysed and samples were analyzed by SDS−PAGE and immunoblotting. We observed labeling of the mutant luciferase with FLAG-Phos, confirming that the reagent is membrane permeable (Figure 1 of the Supporting Information).

To evaluate the exposure of intracellular epitopes to an antibody under nonpermeabilizing conditions, we compared two cell fixatives, paraformaldehyde and methanol. Paraformaldhyde is a protein cross-linking fixative that generally preserves the barrier function of the cellular membrane. On the other hand, methanol denatures the proteins and extracts the lipids, causing the membrane barrier to break down, thereby permeabilizing the cell. To validate the expected behavior of these fixatives in our assay, we expressed the azF-aminoacyl-tRNA synthetase engineered with a C-terminal FLAG peptide epitope tag (azF-RS-FLAG$_{CT}$) that is expressed in the cytosol. A construct lacking the engineered FLAG tag served as a negative control (azF-RS). After the cells had been treated with either fixative, anti-FLAG mAb was used to detect azF-RS in HEK 293T cells expressing either construct. The results indicated that paraformaldehyde was more stringent in eliminating detection of intracellular FLAG peptide epitopes (Figure 2 of the Supporting Information, gray). Additionally, azF-RS without the FLAG tag was not detected under either condition, serving as a negative control. These results suggest that paraformaldehyde would in principle constrain the ELISA detection to epitopes exposed on the cell surface.

Accessibility of an Engineered C-Terminal 1D4 Epitope Tag. Two sites on CCR5, T65 and K229, are predicted to be on the cytoplasmic surface but were labeled in our accessibility screen. To address this observation, we studied three class A GPCRs: CCR5, CXCR4, and rhodopsin. Each receptor clone included a C-terminal 1D4 epitope. The crystal structures of rhodopsin and CXCR4 show that their C-terminal tails are located on the cytoplasmic side of the receptor.[46,47] Each receptor was transfected into HEK 293T cells at two cDNA concentrations (0.1 and 1 μg). Cells were then fixed and subjected to an ELISA with anti-1D4 as the primary antibody (Figure 3 of the Supporting Information). The signals obtained for the permeabilizing methanol fixation correspond to the total level of receptor expression. With paraformaldehyde, the signals were always smaller than those with methanol. However, we would not have expected signals larger than the mock-transfected control, because the C-terminal epitope should be located on the cytoplasmic side of the membrane. Additionally, we observed that an increased level of overexpression of the receptors resulted in an enhanced anti-1D4 ELISA signal with paraformaldehyde fixation. While we do not fully understand the nature of this overexpression artifact, we speculate that membrane ghosts of dead cells with permeable membrane barriers expose the cytoplasmic side of their membranes to the antibodies used in the ELISA. However, these potential artifacts do not limit the utility of the whole-cell-based ELISA to screen for accessible sites for labeling.

Site-Specific Bioorthogonal Labeling and Purification of azF-CCR5 Variants. Here we focus on a preparative strategy to obtain receptors that are bioorthogonally modified at a site-specifically incorporated UAA with a chemical probe. We identified accessible regions in the cell-based screen and selected the sites shown in Figure 4a. We prepared density

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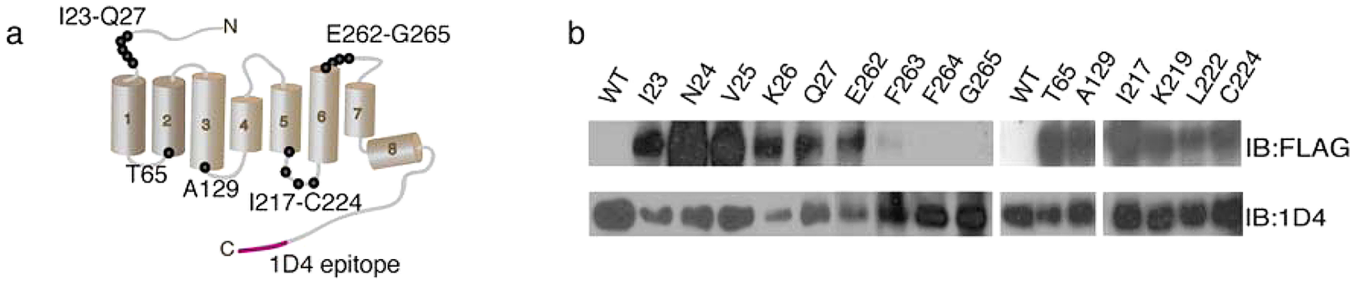

Figure 4. Site-specific bioorthogonal labeling and purification of azF-CCR5 variants. azF-CCR5 was partially enriched by density gradient purification of cellular membranes. These membrane samples were labeled for 16 h with 0.1 mM FLAG-Phos at room temperature. After the samples had been washed and solubilized, the labeled receptor was immuno-affinity purified using the C-terminal 1D4 epitope tag. The samples were analyzed by SDS−PAGE and immunoblotting. (a) CCR5 schematic highlighting positions in which the UAA, azF, was incorporated into the label in membranes. (b) Western blot analysis of labeled and purified azF-CCR5. The top panel shows the receptor probed with the anti-FLAG polyclonal antibody-detected extent of FLAG peptide epitope tagging at each site. The bottom panel shows receptor full-length expression probed with the anti-1D4 antibody against the C-terminal 1D4 epitope tag. As a negative control, wt CCR5 shows no labeling. Similar results were obtained in two replicate experiments. This experiment serves as a model for a future labeling strategy for an attached fluorescent tag instead of the peptide epitope tags.

gradient-purified membranes from cells expressing each azF-CCR5 variant. We incubated these membranes with 0.1 mM FLAG-Phos for 16 h at room temperature. Following labeling, the membranes were washed and solubilized in detergent. CCR5 was immunoaffinity purified from the lysate using the C-terminal 1D4 epitope. Purified samples were separated by SDS−PAGE and immunoblotted with anti-1D4 mAb to show the receptor at the predicted molecular mass of CCR5 (Figure 4b, bottom panel). Anti-FLAG mAb detection of these samples showed a band at the same position demonstrating covalent conjugation of the phosphine reagent to the receptor (Figure 4b, top panel). wt CCR5 showed no detectable incorporation of the label. The results showed variability of the protein content as well as the extent of FLAG incorporation detected for the series of positions tested. The results confirmed several sites identified in the cell-based screen as being suitable candidates for site-specific introduction of a peptide epitope tag into CCR5.

■ DISCUSSION

UAA mutagenesis was developed to encode functional groups with novel chemical, physical, and biological properties into proteins expressed in prokaryotic and eukaryotic systems.[18,19,48] We previously adapted earlier methodologies[22,49] to optimize UAA mutagenesis for low-abundance membrane proteins heterologously expressed in mammalian cells.[23] We also showed the successful incorporation of multiple UAAs and demonstrated their utility in key applications for studying GPCRs.[24−27]

Here we focus on the versatile azido group, which has been reported to be a superior bioorthogonal handle.[29,30] The azido group reacts specifically with phosphine through Staudinger ligation[13,14] or with alkyne reagents through CuAAC[50,51] and SpAAC.[15] Of these, CuAAC reactions achieve the highest labeling efficiency. Cyclooctynes that participate in SpAAC reactions are biocompatible and can overcome the issue of toxicity posed by the Cu(I) catalyst required in CuAAC reactions.[32] However, a recent report shows that SpAAC is not strictly bioorthogonal because reactions with cyclooctynes result in background labeling of cysteine residues in proteins.[35] Despite the susceptibility of phosphines used for the Staudinger ligation to air oxidation[15] and their substoichiometric modification of azF in recombinant proteins[31] and azido sugars,[32] their compatibility with cell-based applications[34] makes them attractive candidates for further study.

In 2000, the Bertozzi group showed the first application of the Staudinger ligation to modify azido-containing glycans on cell surfaces using a biotinylated phosphine derivative.[14] This inspired a decade of new applications of Staudinger ligation to label recombinant proteins with a variety of phosphine derivatives.[13,42] Of these, the FLAG peptide derivative was of particular interest to us. FLAG-Phos was used previously to label an *E. coli* recombinant protein incorporated with azido-bearing methionine analogues in a residue-specific manner.[13] In another study, azido-bearing biotin was used to tag an endogenous biotin acceptor protein (eBAP) by post-translational modification, which was then reacted with FLAG-Phos.[52] While these examples used standard immunoblotting procedures to analyze purified recombinant proteins, we wanted to harness the utility of the peptide epitope tag for antibody detection using a cell-based approach, similar to that demonstrated for detecting FLAG peptide-tagged azido glycans on the cell surface.[53]

We first developed a cell-based strategy to modify site-specifically azF-containing GPCRs by Staudinger ligation in live cells. We then used an antibody-based detection method to interrogate cell surface-expressed receptors. The multistep property of the whole-cell-based ELISA exploits the sequential amplification of the specific signal, allowing detection of low-abundance bioorthogonally modified receptor even in a cellular environment. We measured specific labeling of azF-CCR5 with FLAG-Phos under conditions where wt and AcF-CCR5 did not react at all (Figure 1c), demonstrating that the Staudinger ligation is truly bioorthogonal. We established the reaction conditions required for maximal detection sensitivity of FLAG peptide epitope-tagged CCR5 (Figure 2). Our results for the reaction time course for labeling azF-CCR5 expressed in HEK 293T cells agreed well with results obtained for labeling azido sugars on the surface of Jurkat cells.[14] However, the reported study used biotinylated triarylphosphine at a concentration 10-fold higher than that of the FLAG-Phos used in our experiments.

We next used a semi-high-throughput ELISA to identify positions on CCR5 that were accessible to epitope tagging. The results of the accessibility screen of several azF-CCR5 positions treated with FLAG-Phos indicated that all sites tested on the

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extracellular surface of CCR5, with the exception of F182, located in the second extracellular loop, were suitable candidates for introducing a chemical label (Figure 3b,c). We assume that F182azF is buried within the loop when it adopts the correct conformation required for sensitive epitope recognition by the anti-CCR5 2D7 mAb.[45] Cytoplasmic surface azF residues are also labeled by FLAG-Phos. Therefore, we confirmed that FLAG-Phos is cell membrane permeable by demonstrating labeling of the cytosolic expressed luciferase protein. This observation is not entirely surprising, as other peptide conjugates readily penetrate the cell membrane. Some examples include natural and synthetic cell-penetrating peptides (CPPs) that have found applications in transmembrane delivery and are known as universal intercellular delivery vectors.[54,55] Others include pepducins, designed by attaching a hydrophobic moiety to peptides derived from the intracellular loops of GPCRs.[56,57]

We were intrigued, however, by the unexpected result that modified positions on the cytoplasmic surface of CCR5 were detected under the cell fixation conditions used in the ELISA. The signals obtained in a whole cell ELISA are a collective representation of an ensemble of cells with varying membrane integrity. These include whole cells having intact membranes and those that represent membrane ghosts of dead cells having compromised cellular membrane barriers. In principle, whole cells with intact membranes would permit accessibility of an epitope on the extracellular surface only under appropriate cell fixation conditions. We surmise that membrane ghosts of dead cells allow access of antibodies to epitopes introduced on the cytoplasmic surface. Therefore, one would expect that modified intracellular sites on a receptor would readily be detected in the subset of cells with compromised membrane integrity. Additionally, we note that detection of modified extracellular sites is unaffected by the integrity of the cellular membrane barrier. These observations sparked our interest in investigating the accessibility of the engineered C-terminal 1D4 epitope on our expressed GPCRs. When comparing three different class A GPCRs, we found that an increased level of overexpression of our target GPCR resulted in exposure of the 1D4 epitope tag under nonpermeabilizing fixative treatment. An alternative unorthodox hypothesis for explaining this observation is that a fraction of total receptors expressed at the cell surface adopt a reversed topology as an artifact of overexpression. We stress, however, that these observations do not limit the potential of our assay because our primary objective was to identify sites amenable to peptide epitope tagging on expressed receptors within their cellular bilayer environment. In fact, the ELISA approach provides a platform for screening for potential labeling sites, and as such, false positive results are less detrimental than false negatives.

Finally, we demonstrate the feasibility of a preparative strategy for obtaining purified receptors that are bioorthogonally modified with the FLAG peptide epitope tag. We confirmed that some positive hits obtained in our cell-based accessibility screen were efficiently labeled in membranes (Figure 4b). Interestingly, positions F264 and G265 in the third extracellular loop were poorly labeled in membranes, in contrast to their accessibility to peptide epitope tagging in culture. Labeling receptors in whole cells was performed under cell culturing conditions at 37 °C for 1 h. By contrast, labeling on membranes was conducted at room temperature for 16 h. It is possible that the extracellular loop would adopt an altered local secondary structure via procedures utilized for membrane preparations. On the basis of these results, we stress the importance of using the preparative method to confirm results from the cell-based assay. Moreover, the use of membrane preparations allows attachment of bioorthogonal probes on both topological surfaces of a GPCR. The FLAG peptide epitope was incorporated at positions on the cytoplasmic surface of CCR5 when membrane preparations were used.

It is not always possible to attach a tag to the N-terminal tail of GPCR. Receptors such as PAR1 undergo post-translational modifications in which the N-terminal segment is cleaved,[58] and the N-terminal domain is swapped or undergoes autoproteolysis in adhesion GPCRs.[59] Polytopic proteins such as MDR1 that have both their N- and C-termini located on the cytoplasmic side are good candidates for the site-specific attachment of epitope tags on the extracellular surface.[60] We highlight that the strategy presented in this work allows attachment of a peptide epitope tag at a single modified residue in contrast to inserting the epitope or replacing native sequences with the epitope. This feature is of specific importance with GPCRs because modifying the length and sequence of highly conserved loop segments could dramatically alter function. We propose site-specific insertion of epitope tags that could bind Fab domains to facilitate biophysical or structural analyses.[61]

Membrane proteins of unknown structure are excellent candidates for investigation using this cell-based peptide epitope tagging method. Currently, a combination of standard biochemical methods and sequence-based computational approaches for predicting topology has culminated in databases housing an impressive collection of experimentally and structurally derived topological models.[62,63] Some common methods include the substituted cysteine accessibility method (SCAM)[64] and the use of thiol-specific probes such as monobromobimane to determine topology.[65] However, methods possessing the flexibility and precision of single-codon scanning of transmembrane proteins are limited. Recently, one approach utilized the UAA trifluoromethylphenylalanine, in combination with ^{19}F NMR chemical shift perturbations, to analyze site-specific solvent exposure of an *E. coli* transmembrane protein.[66] We propose that our cell-based approach can find useful applications in determining the general surface topological features of membrane proteins in their native bilayer environment. We observed from the results of our accessibility scan of CCR5 that our method has the potential to discriminate between accessible positions based on the predicted extracellular surface and transmembrane segments of CCR5. We suggest the utility of this method for gaining insight into the secondary structural determinants of a membrane protein of unknown structure.

In summary, we report the feasibility of site-specifically labeling a GPCR in live cells using Staudinger ligation. We envision future in vitro reconstitution of labeled receptors in nanoscale apolipoprotein-bound bilayer (NABB) particles[67] and conducting homogeneous time-resolved fluorescence experiments[3] to study GPCR signaling complexes carrying close proximity probes. We are also establishing the use of alternative chemistries such as Cu-catalyzed and Cu-free azide–alkyne cycloadditions to generate a versatile toolbox for the study of receptor signaling mechanisms of GPCRs at a single-molecule level.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary figures and methods describing the calcium mobilization assay used for functional characterization of receptors. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

AcF, *p*-acetyl-L-phenylalanine; azF, *p*-azido-L-phenylalanine; BzF, *p*-benzoyl-L-phenylalanine; CCR5, C-C chemokine receptor 5; DM, *n*-dodecyl *β*-D-maltoside; ELISA, enzyme-linked immunosorbent assay; FLAG-Phos, FLAG peptide-derivatized triarylphosphine; GPCR, G protein-coupled receptor; HRP, horseradish peroxidase; mAb, monoclonal antibody; RS, aminoacyl-tRNA synthetase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; UAA, unnatural amino acid; wt, wild-type.

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