Copper-free click chemistry in living animals

Pamela V. Chang[a,1], Jennifer A. Prescher[a,1], Ellen M. Sletten[a], Jeremy M. Baskin[a], Isaac A. Miller[a], Nicholas J. Agard[a], Anderson Lo[a], and Carolyn R. Bertozzi[a,b,c,d,2]

[a]Department of Chemistry, [b]Department of Molecular and Cell Biology, and [c]Howard Hughes Medical Institute, University of California, Berkeley, CA 94720; and [d]The Molecular Foundry, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

Edited by M.G. Finn, The Scripps Research Institute, La Jolla, CA, and accepted by the Editorial Board December 9, 2009 (received for review September 25, 2009)

Chemical reactions that enable selective biomolecule labeling in living organisms offer a means to probe biological processes in vivo. Very few reactions possess the requisite bioorthogonality, and, among these, only the Staudinger ligation between azides and triarylphosphines has been employed for direct covalent modification of biomolecules with probes in the mouse, an important model organism for studies of human disease. Here we explore an alternative bioorthogonal reaction, the 1,3-dipolar cycloaddition of azides and cyclooctynes, also known as "Cu-free click chemistry," for labeling biomolecules in live mice. Mice were administered peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz) to metabolically label cell-surface sialic acids with azides. After subsequent injection with cyclooctyne reagents, glycoconjugate labeling was observed on isolated splenocytes and in a variety of tissues including the intestines, heart, and liver, with no apparent toxicity. The cyclooctynes tested displayed various labeling efficiencies that likely reflect the combined influence of intrinsic reactivity and bioavailability. These studies establish Cu-free click chemistry as a bioorthogonal reaction that can be executed in the physiologically relevant context of a mouse.

1,3-dipolar cycloaddition | azide | bioorthogonal ligation | cyclooctyne | glycan

The laboratory mouse is widely regarded as the model organism of choice for studying human pathology because of extensive documentation relating the similarities between human and mouse anatomy, physiology, and cell biology, as well as their genomes (1). Several studies using mouse models have related human disease conditions to altered glycosylation (2). For example, reduced glycosylation of glucose transporter-2 has been shown to impair glucose transport and insulin secretion, producing a condition in mice that is similar to type 2 diabetes in humans (3). Mouse models have also revealed that dysfunctional protein O-GlcNAcylation may contribute to the pathology of Alzheimer's disease (4).

Driven by such observations, researchers are increasingly interested in probing glycosylation, and more generally, carbohydrate metabolism, in mouse models. Toward this goal, Reutter and coworkers demonstrated that unnatural analogs of *N*-acetyl mannosamine that are modified at the *N*-acyl position can be used to probe terminal sialic acid residues within rodents (5). As a means to monitor changes in glycosylation associated with the onset of disease, this approach was later extended to include metabolic labeling with unnatural sugars followed by chemical tagging with probes for fluorescence imaging or affinity capture (6). The unnatural sugar must be outfitted with a bioorthogonal functional group, also termed a "chemical reporter," that possesses unique reactivity with a second functional group installed on the probe reagent (7). The azide is well-suited as a chemical reporter because it adds a small structural perturbation to the underlying sugar, is essentially inert to the cellular environment, and has multiple modes of reactivity (8). The Staudinger ligation, which capitalizes on the electrophilic character of the azide, chemoselectively forms an amide bond between azides and triarylphosphines (9). This reaction has been employed to tag azidosugar-labeled glycoconjugates on cultured cells and in live mice (10, 11).

Despite the utility of the Staudinger ligation, the phosphine reagents have certain liabilities. They are susceptible to oxidation by molecular oxygen, for example, which limits their shelf-life and may also provide a pathway for rapid liver metabolism (12). Moreover, the Staudinger ligation has a relatively sluggish reaction rate, compromising its ability to monitor rapid biological processes in vivo (9). In the context of mouse models, rapid kinetics are important so that the reaction can occur on a faster time scale than metabolic clearance of the probe.

For these reasons, we developed an alternative bioorthogonal reaction modeled on the Huisgen 1,3-dipolar cycloaddition of azides and alkynes, which forms triazole products (13). Our modifications to the classic reaction included situation of the alkyne within a strained cyclooctyne ring (14) as well as the addition of propargylic fluorine atoms (15, 16), two features that significantly accelerated the reaction rate. Another strategy for accelerating the reaction of azides and terminal alkynes involves the use of a Cu catalyst (17, 18), now widely referred to as "click chemistry," but the toxicity of the metal precludes its use in the presence of live cells or organisms. Because cyclooctyne reagents react with azides at rates approaching that of the metal-catalyzed transformation, we refer to these cycloadditions with the term "Cu-free click chemistry." Various cyclooctyne reagents have recently been used to probe glycans on cultured cells (16, 19) and in developing zebrafish (20), as well as protein and phospholipid dynamics in live cells (21, 22).

Here, we evaluate Cu-free click chemistry for its performance in mice with the goal of identifying new reagents that are suitable for bioorthogonal ligations in vivo. We delivered azides to cell-surface sialoglycoconjugates by injecting mice with the metabolic precursor peracetylated *N*-azidoacetylmannosamine (Ac_4ManNAz) (Fig. 1*A*) (11, 23). We then labeled glycoconjugates bearing the corresponding azido sialic acid, SiaNAz, by covalent reaction in vivo with a panel of cyclooctyne-FLAG peptide conjugates (Fig. 1*B*). The labeled biomolecules were probed by ex vivo analysis of cells and tissue lysates. The relative amounts of ligation products observed with different cyclooctynes suggest that both intrinsic reaction kinetics and other properties such as solubility and tissue access govern the efficiency of Cu-free click chemistry in mice. More broadly, Cu-free click chemistry appears to possess the requisite bioorthogonality to achieve specific biomolecule labeling in this important model organism.

Author contributions: P.V.C., J.A.P., J.M.B., and C.R.B. designed research; P.V.C., J.A.P., E.M.S., J.M.B., and I.A.M. performed research; P.V.C., J.A.P., E.M.S., J.M.B., I.A.M., and C.R.B. analyzed data; N.J.A. and A.L. contributed new reagents/analytic tools; and P.V.C. and C.R.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. M.F. is a guest editor invited by the Editorial Board.

[1]P.V.C. and J.A.P. contributed equally to this work.

[2]To whom correspondence should be addressed: Department of Chemistry, B84 Hildebrand Hall #1460, University of California, Berkeley, CA 94720-1460. E-mail: crb@berkeley.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0911116107/DCSupplemental.

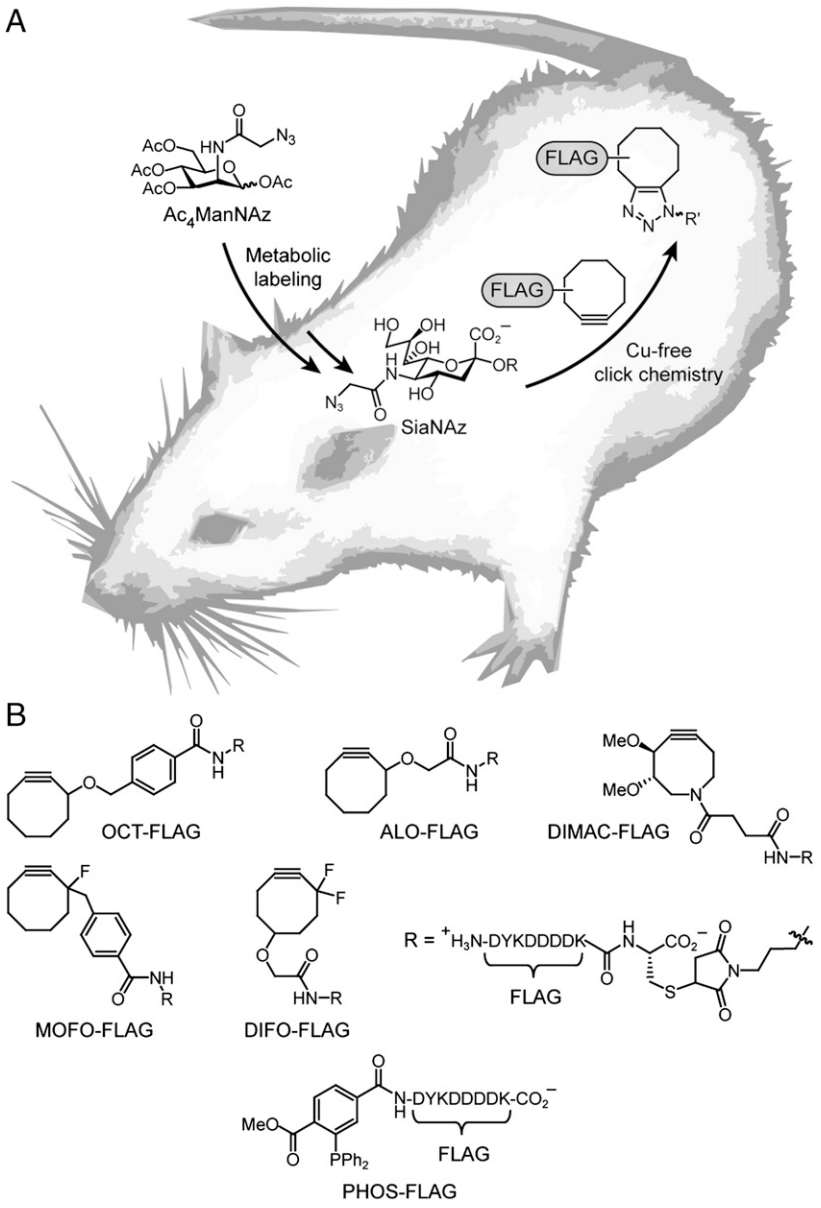

Fig. 1. Cu-free click chemistry in mice. (*A*) Mice were injected with Ac_4ManNAz once daily for 1 wk to allow for metabolic labeling of glycans with SiaNAz. The mice were then injected with a cyclooctyne-FLAG conjugate for in vivo covalent labeling of azido glycans. (*B*) Panel of FLAG conjugates used in this study.

Results and Discussion

Evaluation of Cyclooctyne Reagents on Cultured Cells. Prior to in vivo studies, we noted that our first-generation cyclooctyne, OCT (14), has considerable hydrophobicity (LogS = −5.49*) (24, 25) and accordingly poor water solubility, a property that might compromise its bioavailability. We therefore synthesized an aryl-less cyclooctyne (ALO) lacking the hydrophobic phenyl ring in its linker (15) as well as a highly water-soluble dimethoxy azacyclooctyne (DIMAC) (26). DIMAC possesses a nitrogen atom within the strained ring system to interrupt the hydrophobic surface area as well as two methoxy groups to enhance the compound's polarity. We calculated LogS values* for ALO and DIMAC of −4.22 and −3.35, respectively (24, 25).

To improve the intrinsic reactivity of the cyclooctynes with azides, we previously introduced fluorine atoms at the propargylic position, generating a monofluorinated cyclooctyne (MOFO) (15) and a difluorinated cyclooctyne (DIFO) (16). Recent theoretical studies have attributed the enhanced reactivity of the fluorinated cyclooctynes in cycloadditions with azides to changes in distortion and interaction energies in the transition state of the reaction (27, 28). The relative second-order rate constants of ALO, OCT, DIMAC, MOFO, and DIFO are approximately 1, 2, 3, 4, and 75, respectively, in model reactions (14–16, 26). On this relative scale, the phosphine probe is comparable to OCT.

To facilitate detection of the ligation products, we conjugated a water-soluble FLAG peptide epitope tag (DYKDDDDK) to the cyclooctynes (Fig. 1*B*, synthesis described in *SI Text*). This was achieved by introducing a cysteine residue at the C-terminus of the FLAG peptide sequence that was then coupled to a cyclooctyne-maleimide derivative (*SI Text*).

We next tested the ability of the cyclooctyne-FLAG reagents to label SiaNAz residues within glycoconjugates on cultured

*LogS values were calculated for the Michael addition adducts of methanethiol and the corresponding cyclooctyne maleimide derivatives using the LogS software developed by Tetko et al. (http://www.vcclab.org/lab/alogps/). More negative values indicate poorer water solubility.

cells. Jurkat T cells were incubated with or without Ac_4ManNAz for 3 d. The cells were then treated with a cyclooctyne-FLAG probe or, as a positive control, with a previously described phosphine-FLAG peptide conjugate (PHOS-FLAG, Fig. 1*B*) (29). The cells were analyzed by flow cytometry using a fluorescein isothiocyanate (FITC)-labeled anti-FLAG antibody (FITC-anti-FLAG) (Fig. 2*A*). All reagents labeled the cells in an azide-dependent manner, with DIFO-FLAG generating the strongest signal. Thus, on cultured cells, the rank order of labeling efficiencies matched that of the reagents' intrinsic reactivities.

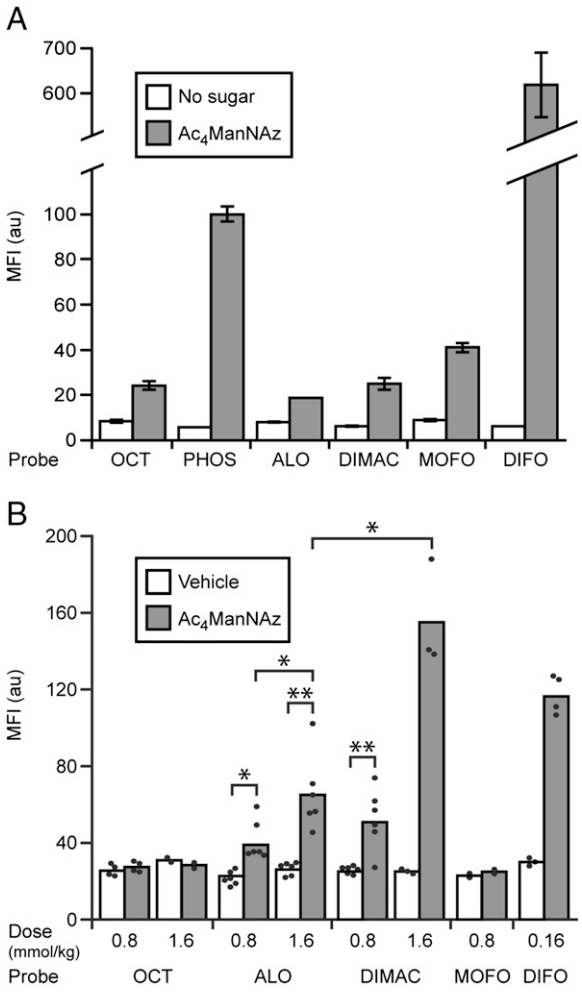

Fig. 2. Chemical tagging of azido glycans in vitro and in vivo with cyclooctyne probes. (*A*) Jurkat cells were incubated with (*Gray Bars*) or without (*White Bars*) Ac_4ManNAz (25 μM) for 3 d. The cells were then labeled with a FLAG conjugate [250 μM of OCT-FLAG (OCT), PHOS-FLAG (PHOS), ALO-FLAG (ALO), DIMAC-FLAG (DIMAC), MOFO-FLAG (MOFO), or DIFO-FLAG (DIFO)] for 1 h at RT. The cells were stained with FITC-conjugated anti-FLAG antibody (FITC-anti-FLAG) and then analyzed by flow cytometry. Error bars represent the standard deviation of the average of three replicate samples. (*B*) Mice were injected with either Ac_4ManNAz (300 mg/kg, i.p., *Gray Bars*) or vehicle (70% DMSO, *White Bars*) once daily for 7 d. On day 8, the mice were injected i.p. with various doses (as indicated) of either OCT-FLAG (OCT), ALO-FLAG (ALO), DIMAC-FLAG (DIMAC), MOFO-FLAG (MOFO), or DIFO-FLAG (DIFO). After 3 h, the mice were euthanized, and splenocytes were isolated, labeled with FITC-anti-FLAG, and analyzed by flow cytometry. Each point represents the average mean fluorescence intensity (MFI) value of three replicate samples from an individual mouse. Each bar represents the average MFI value of splenocytes isolated from separate mice ($n = 3$–11). MFI is in arbitrary units (au). For all in vivo experiments, ex vivo reactions of isolated splenocytes with FLAG conjugates verified the presence of cell-surface azides for all of these probes (*SI Text*). $*P < 0.02$; $**P < 0.008$.

Comparison of Cyclooctyne and Phosphine Probes in Vivo. After confirming the reactivity of the panel of cyclooctynes on cultured cells, we tested whether the cyclooctyne-FLAG conjugates can react with cell-surface SiaNAz residues in vivo. B6D2F1 mice were administered Ac_4ManNAz (300 mg/kg in 70% aqueous DMSO) or vehicle once daily for 7 d in accord with our previous studies of ManNAz metabolism in vivo (11). On day eight, the mice were injected i.p. with a cyclooctyne-FLAG reagent. After 3 h, the mice were euthanized, and the splenocytes were isolated and analyzed for the presence of cycloadducts by flow cytometry. Splenocytes were chosen for analysis based on our previous studies demonstrating the high incorporation levels of SiaNAz into these cells (11).

Interestingly, despite the ability of OCT-FLAG to label azides on cultured cells, splenocytes isolated from mice treated with Ac_4ManNAz and OCT-FLAG (0.8 mmol/kg or 1.6 mmol/kg) did not show any detectable labeling in vivo (Fig. 2*B* and *SI Text*). Subjecting the splenocytes to an additional reaction with the same OCT-FLAG probe ex vivo verified the presence of unreacted cell-surface azides (*SI Text*). The lack of splenocyte labeling by OCT-FLAG could reflect poor access to organs after i.p. injection, perhaps due to limited absorption from the peritoneal cavity into the blood stream. Therefore, we examined serum glycoproteins for the presence of cycloadducts by Western blotting with an anti-FLAG antibody (Fig. 3*A*). No detectable signal was observed, suggesting that OCT-FLAG may not have entered the blood stream after i.p. administration. Alternatively, the compound may be sequestered by nonspecific interactions with serum components or cleared from the blood stream on a time scale that is faster than the rate of the covalent reaction.

Splenocytes from mice treated with both Ac_4ManNAz and either ALO-FLAG or DIMAC-FLAG (both tested at doses of 0.8 mmol/kg and 1.6 mmol/kg) displayed a significant increase in fluorescence relative to those from vehicle mice, indicating that Cu-free click chemistry proceeds in vivo (Fig. 2*B* and *SI Text*). These probes do not possess significantly faster intrinsic kinetics than OCT, yet they react to a greater extent on splenocytes, suggesting that their physical properties contribute to their in vivo reactivities.

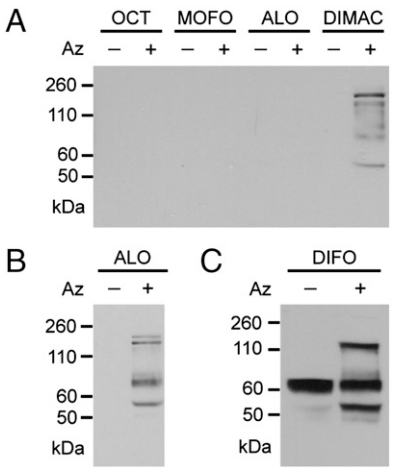

Fig. 3. Chemical tagging of serum glycoproteins in vivo using Cu-free click chemistry. Mice were injected with either Ac_4ManNAz (300 mg/kg, i.p., +Az) or vehicle (70% DMSO, −Az) once daily for 7 d. On day 8, the mice were injected i.p. with various doses of a cyclooctyne-FLAG probe. Serum was collected and analyzed by Western blot with a horseradish peroxidase-anti-FLAG antibody conjugate (HRP-anti-FLAG). (*A*) Samples from mice injected with 0.8 mmol/kg OCT-FLAG (OCT), MOFO-FLAG (MOFO), ALO-FLAG (ALO), DIMAC-FLAG (DIMAC); blot was exposed for 3 s. (*B*) Same blot as (*A*) exposed for 30 s, showing serum glycoproteins labeled with ALO-FLAG. (*C*) Samples from mice injected with 0.16 mmol/kg DIFO-FLAG; exposed for 2 s.

MOFO-FLAG and DIFO-FLAG are more intrinsically reactive than OCT but also less soluble in 70% DMSO, thus mandating the use of lower doses (0.8 mmol/kg and 0.16 mmol/kg, respectively) in the in vivo experiment. Splenocytes isolated from mice treated with both Ac_4ManNAz and MOFO-FLAG did not show any significant labeling in vivo (Fig. 2*B* and *SI Text*). We also examined serum glycoproteins for chemical modification with MOFO-FLAG; however, no labeling was observed (Fig. 3*A*). By contrast, DIFO-FLAG produced significant splenocyte labeling in vivo compared to vehicle-treated mice, even at a dose that was 10-fold lower than the highest dose tested for other cyclooctynes (Fig. 2*B* and *SI Text*). The intrinsic kinetic advantage enjoyed by DIFO in vitro is thus reiterated in vivo, despite its poor solubility properties. We conclude from this comparative study that physical properties as well as inherent reactivities can both contribute to the efficiency of splenocyte labeling in mice.

Encouraged by these results, we sought to compare in vivo Cu-free click chemistry using the best two reagents, DIMAC-FLAG and DIFO-FLAG, with the Staudinger ligation using PHOS-FLAG. In these experiments, mice were injected with either Ac_4ManNAz or vehicle and then either DIMAC-FLAG, DIFO-FLAG, or PHOS-FLAG at the same dose. As shown in Fig. 4, at the same concentrations, PHOS-FLAG (*SI Text*) produced more reaction products on splenocytes than either DIMAC- or DIFO-FLAG, despite the fact that the phosphine's in vitro reaction efficiency is comparable to DIMAC's and 10-fold lower than DIFO's (Fig. 2*A*).

These results may reflect differences in tissue access for the various reagents, a possibility that we addressed by Western blot analysis of serum and tissue lysates from mice treated with Ac_4ManNAz or vehicle, followed by probe-FLAG conjugates. We observed detectable labeling of serum glycoproteins with all of the cyclooctynes except OCT- and MOFO-FLAG as discussed above (Fig. 3). Thus, ALO-, DIMAC-, and DIFO-FLAG have some degree of access to the vasculature. From our panel of three isolated organs, we observed labeling with ALO-FLAG only in the intestines (Fig. 5*A*, overnight exposure). With DIMAC- and DIFO-FLAG, labeling was observed in all of the organs harvested, with the strongest labeling in the intestines, followed by the heart and liver (Fig. 5*B* and *C*, 5 min and 1 h exposure, respectively). The relative intensity of glycoprotein labeling in the intestines may reflect this organ's proximity to the i.p. injection site. For comparative purposes we also probed the same organs for the presence of Staudinger ligation products derived from injected PHOS-FLAG. Again, we observed glycoprotein labeling in all of the organs harvested, with the strongest signal in samples from the intestines, followed by the heart and liver (Fig. 5*D*, 10 s exposure). Thus, PHOS-FLAG appears to access the same organs as DIMAC- and DIFO-FLAG.

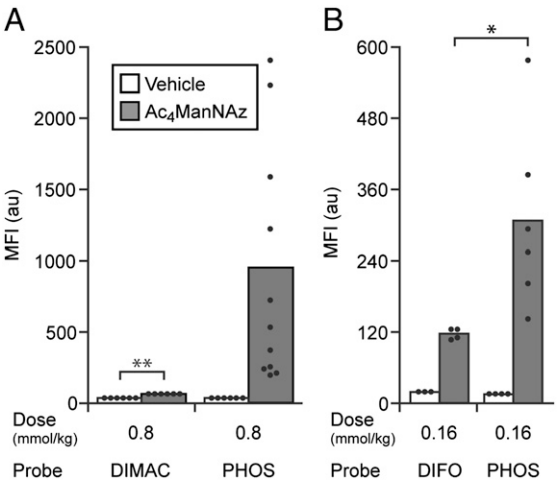

Fig. 4. Comparison of Cu-free click chemistry and the Staudinger ligation for labeling splenocyte cell-surface azides in vivo. Mice were injected with Ac_4ManNAz (300 mg/kg, i.p., *Gray Bars*) or vehicle (70% DMSO, *White Bars*) once daily for 7 d. On day 8, the mice were injected with either (*A*) DIMAC-FLAG (DIMAC, 0.8 mmol/kg, i.p.), (*B*) DIFO-FLAG (DIFO, 0.16 mmol/kg, i.p.), or PHOS-FLAG (PHOS) at the same dose. Three h postinjection of the FLAG conjugates, the mice were euthanized, and the splenocytes were harvested, labeled with FITC-anti-FLAG, and analyzed by flow cytometry. Each point represents the average MFI value of three replicate samples from an individual mouse. Each bar represents the average MFI value of splenocytes isolated from separate mice ($n = 3$–11). $*P < 0.02$; $**P < 0.008$.

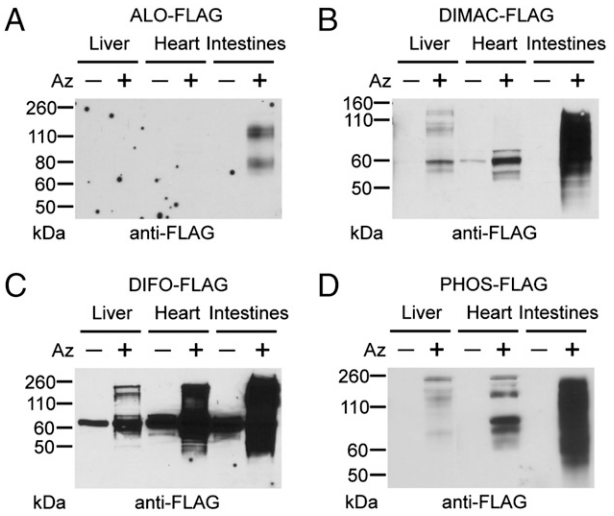

Fig. 5. Cu-free click chemistry and Staudinger ligation products are observed in a variety of tissues in vivo. Mice were injected with Ac_4ManNAz (300 mg/kg, i.p., +Az) or vehicle (70% DMSO, −Az) once daily for 7 d. On day 8, the mice were injected with either (*A*) ALO-FLAG (0.8 mmol/kg, i.p.), blot was exposed overnight; (*B*) DIMAC-FLAG (0.8 mmol/kg, i.p.), blot was exposed for 5 min; (*C*) DIFO-FLAG (0.16 mmol/kg, i.p.), blot was exposed for 1 h; or (*D*) PHOS-FLAG (0.8 mmol/kg, i.p.), blot was exposed for 10 s. Three h postinjection of the FLAG conjugates, the mice were euthanized, and organs (liver, heart, and intestines) were harvested and homogenized. The organ lysates were analyzed by Western blot probing with HRP-anti-FLAG. Each lane represents organ lysate from a single representative mouse.

DIFO-FLAG Binds Mouse Serum Albumin with High Affinity. We noted that the Western blots of organ lysates (Fig. 5*C*) and serum (Fig. 3*C*) from mice treated with vehicle followed by DIFO-FLAG showed a strongly labeled band with an apparent molecular weight around 65 kDa. A similar band, though much more faint, was observed in the Western blot of heart lysates from mice treated with vehicle followed by DIMAC-FLAG (Fig. 5*B*). Thus, DIFO-FLAG and, to a lesser extent, DIMAC-FLAG seem to bind a ubiquitous species in an azide-independent manner. Mouse serum albumin (MSA), with a molecular weight of 68 kDa, was the most obvious candidate (30). MSA is the most abundant serum protein and is known for its role as a carrier protein for small hydrophobic molecules (31).

We tested the MSA-binding activity of DIFO-FLAG by immunoprecipitation of liver lysates derived from Ac_4ManNAz or vehicle-treated mice using an anti-FLAG antibody or isotype control. Western blots of the captured glycoproteins probed using either an anti-FLAG (Fig. 6*A*) or anti-MSA (Fig. 6*B*) antibody confirmed that the species labeled with DIFO-FLAG in an azide-independent manner was indeed MSA. We also immunoprecipitated MSA from liver lysate samples from the same mice using an anti-MSA antibody or isotype control. Western blots of the captured proteins were probed using either an anti-FLAG (Fig. 6*C*) or anti-MSA antibody (Fig. 6*D*) to verify that MSA was indeed modified by the FLAG epitope. It should be noted

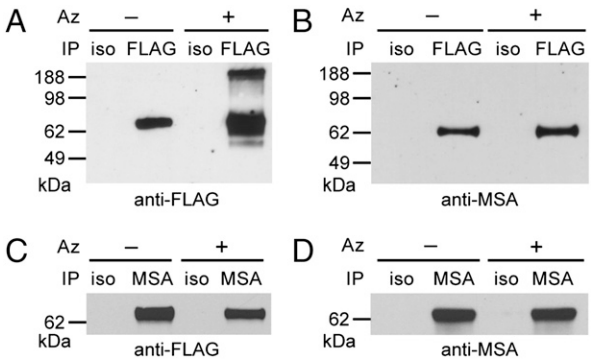

Fig. 6. DIFO-FLAG binds mouse serum albumin (MSA). Liver lysates from mice injected with Ac_4ManNAz (300 mg/kg, i.p., +Az) or vehicle (70% DMSO,–Az) once daily for 7 d, followed by one bolus of DIFO-FLAG (0.16 mmol/kg, i.p.) on day 8, were immunoprecipitated with (*A–B*) an anti-FLAG antibody (FLAG) or an isotype control (iso), or (*C–D*) an anti-MSA antibody (MSA) or an isotype control (iso). The samples were analyzed by Western blot probing for (*A*) and (*C*) FLAG or (*B*) and (*D*) MSA.

that the Western blots in Fig. 6 reflect samples that were exposed to both 1 M urea and β-mercaptoethanol and were boiled for 10 min before being run on a reducing, denaturing gel. The persistence of MSA labeling after these treatments suggests that the interaction might be covalent. MSA has two free cysteine residues that are subject to covalent modification by other metabolites (32). It is possible that nonspecific binding to the DIFO core, a highly hydrophobic moiety, facilitates covalent interactions with cysteine or other nucleophilic residues.

Conclusion

Cu-free click chemistry has been previously used to monitor azidosugars (16, 33), proteins bearing azido amino acids (34, 35), lipids (22), and site-specifically labeled proteins (21) in live cells. In this paper, we demonstrated that Cu-free click chemistry using a variety of cyclooctyne probes can be employed to label azidosugars in the physiologically relevant context of a mouse. Cyclooctynes thereby join the ranks of a privileged few reagents that possess the requisite bioorthogonality to find their covalent reaction partners in animals. Most of these reagents, however, require noncovalent complexation with an enzyme target to deliver a covalent warhead to a nucleophilic residue nearby (36, 37). Only the Staudinger ligation and Cu-free click chemistry reagents possess sufficient bioorthogonality for direct covalent labeling of their targets in vivo. The ability to perform these chemical reactions in living animals could enable therapeutic targeting or molecular imaging of changes in metabolism in mouse models of human disease.

Among the cyclooctynes tested, DIFO-FLAG, which possesses the best intrinsic reactivity with azides, was the most efficient reagent for labeling serum or tissue-resident glycoproteins. DIMAC- and ALO-FLAG also afforded detectable labeling, though at a lower level. Their advantage over OCT- and MOFO-FLAG may reflect their superior water solubility. It should be noted, however, that PHOS-FLAG, a reagent with lower inherent reactivity than DIFO, gave the most robust labeling of splenocytes after the 3 h in vivo reaction. DIFO's bioavailability may be compromised by significant binding to serum albumin. Thus, optimization of bioorthogonal reagents for in vivo applications may require both consideration of their intrinsic reactivities as well as their pharmacokinetic properties.

Analysis of the detailed biodistributions and serum residence times of the above reagents will be an interesting extension of this work. Moreover, pathways of metabolic clearance will be important to characterize in the design of optimal bioorthogonal reagents. Literature precedents suggest that phosphines (12) and terminal alkynes (38) can be metabolized in the liver by cytochrome P450 enzymes. Internal alkynes also may be oxidized by P450 enzymes, though their routes of metabolism are less well characterized (39). The metabolism of cyclooctynes has not been explored, offering another interesting future direction.

Materials and Methods

Chemical Synthesis. Details describing the synthesis and characterization of all compounds can be found in *SI Text*.

Cell Culture. Jurkat (human T cell lymphoma) cells and isolated splenocytes were maintained in a 5% CO_2, water-saturated atmosphere and grown in RPMI media 1640 supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (0.1 mg/ml). Cell densities were maintained between 1×10^5 and 2×10^6 cells per ml.

Labeling of Cultured Cells with FLAG Conjugates and Flow Cytometry Analysis. Metabolic labeling of Jurkat cells with Ac_4ManNAz and chemical labeling using FLAG conjugates were performed essentially as previously described (11). Details can be found in *SI Text*.

Mice. Wild-type B6D2F1 mice (aged 5–8 wk) were purchased from The Jackson Laboratory or Charles River Laboratories. Animals were handled in accordance with Animal Use Protocol R234-0609B (approved by the Animal Care and Use Committee at the University of California, Berkeley).

Compound Administration. Ac_4ManNAz (29), PHOS-FLAG (29), OCT (14), ALO (15), MOFO (15), DIMAC (26), DIFO-maleimide (16), and DIFO-FLAG (16) were synthesized according to previously published procedures. Synthetic methods and spectral data for new compounds are provided in the *SI Text*. B6D2F1 mice were administered Ac_4ManNAz (300 mg/kg in 70% DMSO from a 116 mM stock solution) or vehicle (70% DMSO) i.p. once daily for 7 d. Twenty-four h after the final Ac_4ManNAz bolus, mice were injected i.p. with either PHOS-FLAG (0.16 mmol/kg or 0.8 mmol/kg in 70% DMSO or water from a 26.7 mM or 133 mM stock solution, respectively), OCT-FLAG (0.8 mmol/kg or 1.6 mmol/kg in 70% DMSO from a 133 mM or 267 mM stock solution, respectively), ALO-FLAG (0.8 mmol/kg or 1.6 mmol/kg in 70% DMSO from a 133 mM or 267 mM stock solution, respectively), DIMAC-FLAG (0.8 mmol/kg, and 1.6 mmol/kg in water from a 133 mM or 267 mM stock solution, respectively), MOFO-FLAG (0.8 mmol/kg in 70% DMSO from a 133 mM stock solution), or DIFO-FLAG (0.16 mmol/kg in 70% DMSO from a 26.7 mM stock solution). All doses of azidosugar, vehicle, and FLAG conjugates were administered in approximately 150 μl of 70% DMSO or water. Three h postinjection of the FLAG conjugates, the mice were euthanized, and a panel of organs was harvested.

Splenocyte Analysis After Cu-Free Click Chemistry in Vivo. Splenocytes from mice treated first with Ac_4ManNAz or vehicle followed by the appropriate FLAG conjugate were isolated and probed for the presence of cell-surface FLAG epitopes using a previously reported protocol (11). Briefly, isolated splenocytes were incubated directly with FITC-anti-FLAG in labeling buffer (PBS + 1%FBS, 1:900 dilution from Sigma stock) for 30 min on ice. The cells were then washed once with labeling buffer and analyzed by flow cytometry.

Labeling of Splenocyte Cell-Surface Azides ex Vivo. Splenocytes isolated as described above were further reacted ex vivo with OCT-FLAG, ALO-FLAG, MOFO-FLAG, or PHOS-FLAG to probe for the presence of unreacted azides. Briefly, splenocytes were incubated with either PHOS-FLAG or one of the cyclooctyne-FLAG conjugates (250 μM) for 1 h at room temperature in labeling buffer. The cells were then rinsed three times with labeling buffer, treated with FITC-anti-FLAG for 30 min on ice, rinsed with labeling buffer once, and analyzed by flow cytometry.

Western Blot Analysis of Serum Glycoproteins. Whole blood was collected from anesthetized mice injected first with either Ac_4ManNAz or vehicle and then PHOS-FLAG or a cyclooctyne-FLAG conjugate by cardiac puncture from the closed thorax. The samples were allowed to clot, and the serum was then isolated by removal of the agglutinated red blood cells and leukocytes. The samples were then centrifuged (13500 × g for 10 min) to remove residual cell debris, and the supernatant was collected and diluted with lysis buffer (150 mM NaCl, 1.0% NP-40, 20 mM Tris-HCl, 1 mM EDTA, pH 7.4) containing protease inhibitors (Complete, Roche). Protein concentrations were determined using the *DC* protein assay kit (BioRad). The samples (50 μg of protein per lane) were analyzed by Western blot, probing with a horseradish

peroxidase-anti-FLAG antibody conjugate (HRP-anti-FLAG) as previously described (11).

Lysis of Murine Organs and Western Blot Analysis. Organs (liver, heart, and intestines) harvested from mice injected first with either Ac_4ManNAz or vehicle and then PHOS-FLAG or a cyclooctyne-FLAG conjugate were rinsed with cold PBS and minced. The organs were then transferred into 1.5 ml of lysis buffer (150 mM NaCl, 1.0% NP-40, 20 mM Tris-HCl, 1 mM EDTA, pH 7.4) containing protease inhibitors (Complete, Roche) and homogenized using a Dounce homogenizer. The lysates were centrifuged (13500 × g for 10 min) to remove cell debris, and the supernatant was collected. Protein concentrations were determined using the *DC* protein assay kit (BioRad). The samples (100 μg of protein per lane) were analyzed by Western blot, probing with HRP-anti-FLAG as previously described (11).

Immunoprecipitation of FLAG-Labeled Liver Lysates to Probe for MSA. Liver lysate (10 mg of protein) from mice treated with either Ac_4ManNAz or vehicle and subsequently injected with DIFO-FLAG were prepared as described above and incubated with 60 μg of M2 anti-FLAG antibody (Sigma) or isotype control (mouse IgG1 isotype, Sigma) in 10 ml of lysis buffer containing protease inhibitorx (Complete, Roche) at 4 °C with rotation overnight. Ultralink Immobilized Protein A/G (60 μl, Pierce) was added, and the sample was rotated for 2 h at room temperature. At this point, the resin was pelleted by centrifugation (1000 × g for 1 min), and the supernatant was discarded. Wash buffer A (750 μl of 50 mM Tris, 300 mM NaCl, 1% Triton X-100, pH 7.4) was added. The sample was pelleted on a benchtop microfuge and the supernatant was discarded. A second wash with 750 μl of wash buffer A was performed, followed by two washes of 750 μl each with wash buffer B (50 mM Tris, 1.3 M NaCl, 1% Triton X-100, pH 7.4), and wash buffer C (50 mM Tris, 1.3 M NaCl, 1 M urea, 1% Triton X-100, pH 7.4). The beads were then diluted with 1X SDS gel loading buffer (BioRad) containing β-mercaptoethanol, boiled for 10 min, and analyzed by Western blot, using HRP-anti-FLAG (1 : 1000 dilution from Sigma stock) to probe for FLAG (1/5 of the total elution) or goat anti-MSA (affinity-purified polyclonal, 1 : 5000 dilution from Bethyl Laboratories stock), followed by donkey anti-goat-HRP (1 : 5000 dilution from Santa Cruz Biotechnology stock) to probe for MSA (4/5 of the total elution).

Immunoprecipitation of MSA from Liver Lysates to Probe for the FLAG Epitope. MSA was immunoprecipitated as previously described (32). Briefly, liver lysate (10 mg of protein) from mice treated with either Ac_4ManNAz or vehicle and subsequently injected with DIFO-FLAG were prepared as described above. The samples were precleared with Ultralink Immobilized Protein A/G (50 μl, Pierce) by rotation for 1 h at 4 °C in 10 ml of 50 mM sodium phosphate, pH 7.4 (immunoprecipitation buffer), followed by centrifugation to pellet the beads (3700 × g for 5 min) and collection of the supernatant. In separate tubes, anti-MSA or an isotype control antibody (goat anti-mouse IgG) (15 μg) was bound to a separate sample of Ultralink Immobilized Protein A/G (50 μl) by rotation for 1 h at 4 °C in 10 ml of immunoprecipitation buffer. The antibody-bound beads were rinsed once with 5 ml of immunoprecipitation buffer, and the precleared lysate was added to the antibody-bound beads and rotated overnight at 4 °C. Then the beads were pelleted (3700 × g for 5 min), rinsed 3 times with 1 ml of immunoprecipitation buffer, and eluted by boiling for 10 min in 1X SDS gel loading buffer (BioRad). One quarter of the sample was analyzed by Western blot, probing with goat anti-MSA (affinity-purified polyclonal, 1 : 5000 dilution from Bethyl Laboratories stock) followed by donkey anti-goat-HRP (1 : 5000 dilution of from Santa Cruz Biotechnology stock), and the remainder of the elution was analyzed by Western blot, probing with HRP-anti-FLAG (1 : 1000 dilution from Sigma stock).

ACKNOWLEDGMENTS. We thank Danielle Dube, Sarah Hubbard, Julian Codelli, and Bryan Dickinson for technical assistance and Scott Laughlin for graphical assistance. This work was supported by National Institutes of Health Grant GM058867. P.V.C. was supported by National Science Foundation and American Chemical Society Division of Medicinal Chemistry predoctoral fellowships. J.A.P. was supported by a Howard Hughes Medical Institute predoctoral fellowship. E.M.S. was supported by an American Chemical Society Division of Organic Chemistry predoctoral fellowship. J.M.B. was supported by National Science Foundation and National Defense Science and Engineering predoctoral fellowships.

1. Peters LL, et al. (2007) The mouse as a model for human biology: A resource guide for complex trait analysis. *Nat Rev Genet*, 8:58–69.
2. Ohtsubo K, Marth JD (2006) Glycosylation in cellular mechanisms of health and disease. *Cell*, 126:855–867.
3. Ohtsubo K, et al. (2005) Dietary and genetic control of glucose transporter 2 glycosylation promotes insulin secretion in suppressing diabetes. *Cell*, 123:1307–1321.
4. Hart GW, Housley MP, Slawson C (2007) Cycling of O-linked beta-N-acetylglucosamine on nucleocytoplasmic proteins. *Nature*, 446:1017–1022.
5. Kayser H, et al. (1992) Biosynthesis of a nonphysiological sialic acid in different rat organs, using N-propanoyl-D-hexosamines as precursors. *J Biol Chem*, 267: 16934–16938.
6. Prescher JA, Bertozzi CR (2006) Chemical technologies for probing glycans. *Cell*, 126:851–854.
7. Prescher JA, Bertozzi CR (2005) Chemistry in living systems. *Nat Chem Biol*, 1:13–21.
8. Sletten EM, Bertozzi CR (2009) Bioorthogonal chemistry: Fishing for selectivity in a sea of functionality. *Angew Chem Int Ed Engl*, 48:6974–6998.
9. Lin FL, Hoyt HM, van Halbeek H, Bergman RG, Bertozzi CR (2005) Mechanistic investigation of the Staudinger ligation. *J Am Chem Soc*, 127:2686–2695.
10. Kohn M, Breinbauer R (2004) The Staudinger ligation—A gift to chemical biology. *Angew Chem Int Ed Engl*, 43:3106–3116.
11. Prescher JA, Dube DH, Bertozzi CR (2004) Chemical remodelling of cell surfaces in living animals. *Nature*, 430:873–877.
12. Wiley RA, Sternson LA, Sasame HA, Gillette JR (1972) Enzymatic oxidation of diphenylmethylphosphine and 3-dimethylaminopropyldiphenylphosphine by rat liver microsomes. *Biochem Pharmacol*, 21:3235–3247.
13. Huisgen R (1963) 1.3-dipolare cycloadditionen—Ruckschau und Ausblick. *Angew Chem Int Ed*, 75:604–637.
14. Agard NJ, Prescher JA, Bertozzi CR (2004) A strain-promoted [3 + 2] azide-alkyne cycloaddition for covalent modification of biomolecules in living systems. *J Am Chem Soc*, 126:15046–15047.
15. Agard NJ, Baskin JM, Prescher JA, Lo A, Bertozzi CR (2006) A comparative study of bioorthogonal reactions with azides. *ACS Chem Biol*, 1:644–648.
16. Baskin JM, et al. (2007) Copper-free click chemistry for dynamic in vivo imaging. *Proc Natl Acad Sci USA*, 104:16793–16797.
17. Rostovtsev VV, Green LG, Fokin VV, Sharpless KB (2002) A stepwise huisgen cycloaddition process: Copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angew Chem Int Ed Engl*, 41:2596–2599.
18. Tornoe CW, Christensen C, Meldal M (2002) Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *J Org Chem*, 67:3057–3064.
19. Ning X, Guo J, Wolfert MA, Boons GJ (2008) Visualizing metabolically labeled glycoconjugates of living cells by copper-free and fast huisgen cycloadditions. *Angew Chem Int Ed Engl*, 47:2253–2255.
20. Laughlin ST, Baskin JM, Amacher SL, Bertozzi CR (2008) In vivo imaging of membrane-associated glycans in developing zebrafish. *Science*, 320:664–667.
21. Fernandez-Suarez M, et al. (2007) Redirecting lipoic acid ligase for cell surface protein labeling with small-molecule probes. *Nat Biotechnol*, 25:1483–1487.
22. Neef AB, Schultz C (2009) Selective fluorescence labeling of lipids in living cells. *Angew Chem Int Ed Engl*, 48:1498–1500.
23. Laughlin ST, Bertozzi CR (2007) Metabolic labeling of glycans with azido sugars and subsequent glycan-profiling and visualization via Staudinger ligation. *Nat Protoc*, 2:2930–2944.
24. Tetko IV, Tanchuk VY, Kasheva TN, Villa AE (2001) Internet software for the calculation of the lipophilicity and aqueous solubility of chemical compounds. *J Chem Inf Comput Sci*, 41:246–252.
25. Tetko IV, Tanchuk VY, Kasheva TN, Villa AE (2001) Estimation of aqueous solubility of chemical compounds using E-state indices. *J Chem Inf Comput Sci*, 41:1488–1493.
26. Sletten EM, Bertozzi CR (2008) A hydrophilic azacyclooctyne for Cu-free click chemistry. *Org Lett*, 10:3097–3099.
27. Ess DH, Jones GO, Houk KN (2008) Transition states of strain-promoted metal-free click chemistry: 1,3-dipolar cycloadditions of phenyl azide and cyclooctynes. *Org Lett*, 10:1633–1636.
28. Bach RD (2009) Ring strain energy in the cyclooctyl system. The effect of strain energy on [3 + 2] cycloaddition reactions with azides. *J Am Chem Soc*, 131:5233–5243.
29. Laughlin ST, et al. (2006) Metabolic labeling of glycans with azido sugars for visualization and glycoproteomics. *Methods Enzymol*, 415:230–250.
30. Mano N, Sato K, Goto J (2006) Specific affinity extraction method for small molecule-binding proteins. *Anal Chem*, 78:4668–4675.
31. Peters T (1996) *All About Albumin* (Academic, San Diego).
32. Cheng L, et al. (2008) Covalent binding of the nitroso metabolite of sulfamethoxazole is important in induction of drug-specific T-cell responses in vivo. *Mol Pharmacol*, 73:1769–1775.
33. Poloukhtine AA, Mbua NE, Wolfert MA, Boons GJ, Popik VV (2009) Selective labeling of living cells by a photo-triggered click reaction. *J Am Chem Soc*, 131:15769–15776.
34. Link AJ, Vink MK, Tirrell DA (2004) Presentation and detection of azide functionality in bacterial cell surface proteins. *J Am Chem Soc*, 126:10598–10602.
35. Link AJ, Tirrell DA (2003) Cell surface labeling of Escherichia coli via copper(I)-catalyzed [3 + 2] cycloaddition. *J Am Chem Soc*, 125:11164–11165.
36. Edgington LE, et al. (2009) Noninvasive optical imaging of apoptosis by caspase-targeted activity-based probes. *Nat Med*, 15:967–973.
37. Tsukiji S, Miyagawa M, Takaoka Y, Tamura T, Hamachi I (2009) Ligand-directed tosyl chemistry for protein labeling in vivo. *Nat Chem Biol*, 5:341–343.
38. Ortiz de Montellano PR (1985) *Bioactivation of Foreign Compounds* (Academic, Orlando).
39. Ortiz de Montellano PR, Kunze KL (1980) Self-catalyzed inactivation of hepatic cytochrome P-450 by ethynyl substrates. *J Biol Chem*, 255:5578–5585.