ACS chemical biology

Letters

pubs.acs.org/acschemicalbiology

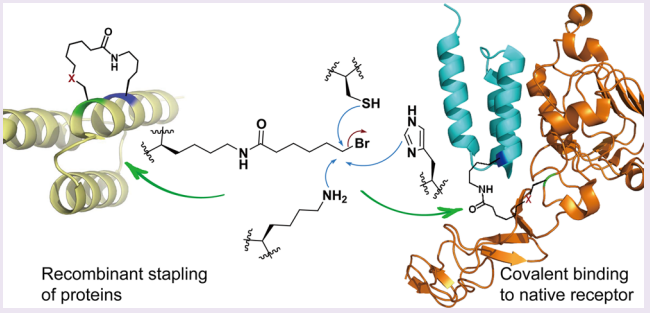
Genetically Encoding an Electrophilic Amino Acid for Protein Stapling and Covalent Binding to Native Receptors

Xiao-Hua Chen,[†] Zheng Xiang,[†] Ying S. Hu,[‡] Vanessa K. Lacey,[†] Hu Cang,[‡] and Lei Wang*[,†]

[†]The Jack H. Skirball Center for Chemical Biology and Proteomics and [‡]Waitt Advanced Biophotonics Center, The Salk Institute for Biological Studies, 10010 N. Torrey Pines Road, La Jolla, California 92037, United States

Supporting Information

ABSTRACT: Covalent bonds can be generated within and between proteins by an unnatural amino acid (Uaa) reacting with a natural residue through proximity-enabled bioreactivity. Until now, Uaas have been developed to react mainly with cysteine in proteins. Here we genetically encoded an electrophilic Uaa capable of reacting with histidine and lysine, thereby expanding the diversity of target proteins and the scope of the proximity-enabled protein cross-linking technology. In addition to efficient cross-linking of proteins inter- and intramolecularly, this Uaa permits direct stapling of a protein α-helix in a recombinant manner and covalent binding of native membrane receptors in live cells. The target diversity, recombinant stapling, and covalent targeting of endogenous proteins enabled by this versatile Uaa should prove valuable in developing novel research tools, biological diagnostics, and therapeutics by exploiting covalent protein linkages for specificity, irreversibility, and stability.



The ability to selectively generate covalent bonds in proteins would enable new avenues for studying protein functions and engineering protein properties, which will find broad applications in basic and synthetic biology. We recently introduced a general strategy of creating new covalent bonds in proteins, in which an unnatural amino acid (Uaa) is designed to react with a natural amino acid via proximity-enabled bioreactivity.[1] The reactivity of the Uaa is fine-tuned so that it reacts only with the target natural amino acid residue when the two amino acids are brought into proximity, either by interprotein interaction or by intraprotein folding and conformational change. Using this strategy, covalent bonds have been generated between proteins, allowing irreversible binding of proteins and mapping of ligand−receptor interactions in situ,[2] and within proteins, increasing the photon output of fluorescent proteins and the thermostability of an affibody.[1,3]

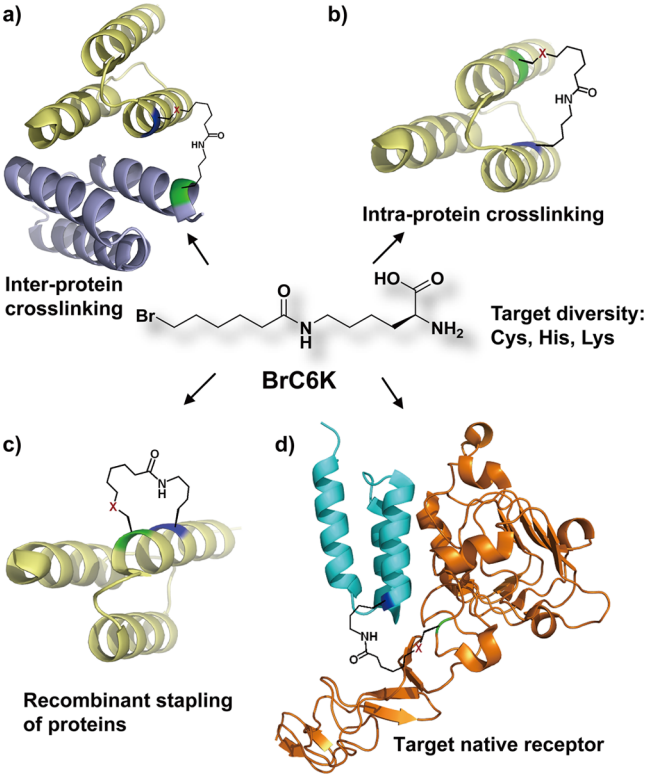
Initial efforts in developing proximity-enabled bioreactivity have been focused on targeting the Cys residue,[1] the sulfhydryl group of which has the highest nucleophilicity among natural amino acid side chains. Although Cys plays a crucial role in disulfide bond formation and catalysis in a variety of proteins, other amino acids containing hydrophilic side chains are often found at protein surfaces and interfaces where a Cys may be absent.[4] The ability to target residues other than Cys for covalent bond generation would dramatically expand the diversity of proteins applicable by proximity-enabled bioreactivity. In addition, all of the Uaas developed to target Cys are derivatives of phenylalanine,[1,3] containing a rigid aromatic group in the side chain, which prefers a hydrophobic microenvironment and may limit flexibility in bond formation. A different amino acid scaffold for the Uaa should complement these limitations and broaden the scope of possible applications.

Herein we report the design and genetic incorporation of an electrophilic amino acid using an alkyl scaffold, which enables covalent bond formation with a nearby His or Lys residue. Proximity-enabled bioreactivity can now be generally expanded to target natural amino acids other than Cys (Scheme 1). Using this Uaa we demonstrate a recombinant stapling technology capable of stapling the secondary structures of proteins and covalently target the endogenous membrane receptor in live cells.

The side chains of His and Lys are weak nucleophiles, which rarely react under the mild conditions necessary for cross-linking native proteins. However, the ε-NH_2 of Lys has been shown to react with the side chain carbonyl of Asn or Asp to form isopeptide bonds when catalyzed by a glutamic acid residue during protein folding.[5] We thus hypothesized that a Uaa bearing an electrophilic group, when its structure and reactivity is tuned appropriately, might react with a nearby Lys or His residue. On the basis of results of haloalkane Uaas reacting with Cys,[3] we decided to test the alkyl bromide, which has strong reactivity toward Cys. We reasoned that a linear alkyl side chain, which is more flexible than the aromatic group

Received: June 6, 2014
Accepted: July 9, 2014
Published: July 10, 2014

Scheme 1. A Versatile Uaa BrC6K[a]



[a]Through proximity-enabled bioreactivity, BrC6K is able to target three natural amino acid residues to generate covalent linkages for protein crosslinking between (a) and within (b) proteins, for recombinant protein stapling (c), and for covalent binding to native receptors (d).

previously used, may provide orientation flexibility to facilitate the reaction. On the basis of these considerations, we designed and synthesized the Uaa (*S*)-2-amino-6-(6-bromohexanamido)-hexanoic acid (BrC6K) with a long linear alkyl side chain and terminal bromide as the leaving group (Scheme 1). Bromide was chosen over iodide for better stability.

To evolve an aminoacyl-tRNA synthetase specific for BrC6K, we generated mutant synthetase libraries by randomizing 6 or 7 residues in the active site of the *Methanosarcina mazei* PylRS (MmPylRS)[6,7] and performed three rounds of positive and two rounds of negative selections using procedures previously described.[6−17] After the third positive selection, 36 green fluorescent colonies were obtained and subsequently screened by streaking on minimal-medium plates supplemented with chloramphenicol (60 μg mL^{-1}) and in the presence or absence of 1 mM BrC6K. All of these colonies showed BrC6K-dependent survival in chloramphenicol (up to 160 μg mL^{-1} in rich medium). DNA sequencing of eight individual colonies revealed that, despite codon differences at the mutation sites, they all converged on one mutant on the protein level containing mutation Y384F and named MmBrC6KRS for clarity. The mutation of Y384F was previously found to increase the aminoacylation rate of PylRS and to accommodate several Lys analogues.[11]

To evaluate the translational efficiency and fidelity of MmBrC6KRS, we expressed the sperm whale myoglobin gene (Myo-4TAG-His6, encoding a TAG codon at position 4 and a C-terminal His×6 tag) together with the $tRNA^{Pyl}_{CUA}$-MmBrC6KRS in *E. coli*. SDS-PAGE analysis of the purified protein showed that full-length myoglobin (Supplementary Figure S1a) was obtained in good yield (ca. 6 mg L^{-1}) in the presence of 2 mM BrC6K but was undetectable in the absence of BrC6K. The purified myoglobin was analyzed by electrospray ionization Fourier transform ion trap mass spectrometry (ESI-FTMS) (Supplementary Figure S1b). High resolution monoisotopic masses indicate that only BrC6K was incorporated at the TAG-encoded position in myoglobin and that BrC6K was stable during protein synthesis in *E. coli* and throughout the purification process.

To investigate the Proximity-Enabled Protein Cross-linking (PEPC) efficiency of BrC6K in comparison to that of the previously developed Uaas containing aromatic groups (BetY and IetY, Figure 1a),[3] we chose to cross-link the Z_{SPA} affibody

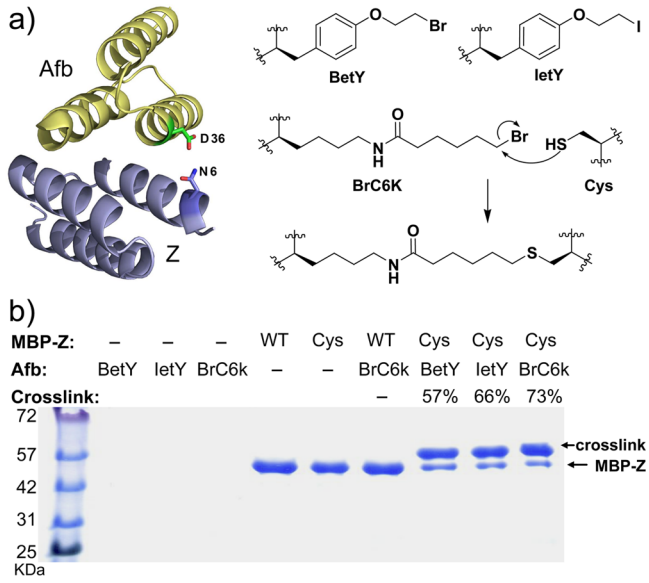


Figure 1. BrC6K reacts with Cys efficiently in intermolecular PEPC. (a) Crystal structure of the Afb-Z complex (PDB ID: 1LP1) showing Asp36 in the Afb and Asn6 in the Z protein, to be substituted by the Uaa (BrC6K, BetY, or IetY) and Cys, respectively. (b) SDS-PAGE analysis of Afb−Z cross-linking. The identities of residue 6 of the Z protein in MBP−Z and residue 36 of the Afb are indicated. Afb mutant proteins ran at ∼8 kDa on SDS-PAGE (Supplementary Figure S2a). The cross-linking efficiencies were measured by four independent cross-linking experiments (Supplementary Figure S3).

(Afb) with its substrate Z protein.[18] We fused the Z protein to the maltose binding protein (MBP) for clear separation by molecular weight. The Uaa and Cys were incorporated into the Afb (site 36) and MBP-Z (site 6), respectively, at two proximal sites of the binding interface.[1,18] The purified Afb (D36Uaa) proteins were incubated with the purified MBP-Z(N6C) in identical reaction conditions. From denatured SDS-PAGE analysis of the reaction mixture (Figure 1b, Supplementary Figure S3), we found that BrC6K showed higher PEPC efficiency (73%) than BetY (57%), which has the same leaving group as BrC6K. Moreover, even though IetY contains the more reactive alkyl iodide, BrC6K still showed higher cross-linking efficiency than IetY (66%). These results indicate that the long alkyl flexible side chain of BrC6K enhanced PEPC efficiency when targeting Cys.

The increased PEPC efficiency of BrC6K with Cys encouraged us to explore whether BrC6K could cross-link with other natural amino acid residues. Taking into

dx.doi.org/10.1021/cb500453a | *ACS Chem. Biol.* 2014, 9, 1956−1961

consideration the nucleophilicity and pK_a of each potential side chain, we reasoned that His and Lys would be likely targets. To test intramolecular PEPC, two proximal sites, F30 and E47, in the same Afb protein[18] were chosen for incorporating the Uaa and Lys/His/Cys, respectively (Figure 2a). Each mutant protein was obtained in good yield from *E. coli* and showed a clear band at the same position compared to each other in SDS-PAGE (Figure 2b–d). ESI-FTMS analyses of the mutant proteins Afb-30Uaa-47C, Afb-30Uaa-47H, and Afb-30Uaa-47K clearly showed covalent bond formation between the Uaa and the targeted natural residue in each protein. As expected, Cys was the most reactive toward BrC6K due to the high nucleophilicity of the sulfhydryl group, and the covalent cross-linking was spontaneous (pH 7.4) and complete (Figure 2b). BrC6K formed a covalent bond with His after the protein was incubated at pH 8.0 or pH 8.8 for 6 h (37 °C) (Figure 2c). Based on the relative abundance of the cross-linked versus non-cross-linked peaks, the intraprotein cross-linking yield of His with BrC6K was 23% at pH 8.0 (Supplementary Figure S4). A notable increase in cross-linking was observed when the identical protein was incubated at pH 8.8 (~50% yield, Figure 2c), at which a higher percentage of the His side chain would be in the deprotonated state for nucleophilic reaction. In addition, BrC6K was also able to covalently cross-link with Lys at pH 8.8 (Figure 2d, ~12% yield). The yield was lower than that with His, for which the higher pK_a of the Lys side chain may play a role. In contrast to BrC6K, BetY and IetY could target His or Lys with only very low efficiencies (1–2%) in the intraprotein cross-linking of Afb under the identical conditions (Supplementary Figure S5–8). These results demonstrate that Uaa BrC6K enabled significant reactivity toward His and Lys.

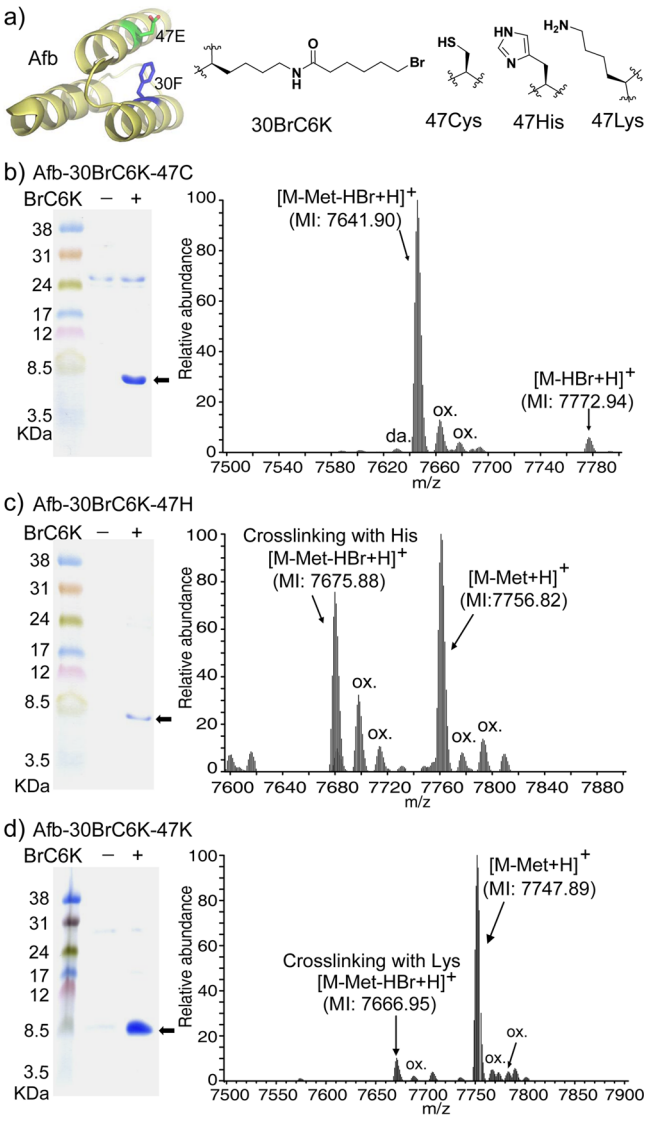


Figure 2. BrC6K is able to target His and Lys in addition to Cys in intramolecular PEPC. (a) Crystal structure of the Afb (PDB ID: 1LP1) showing the two proximal sites F30 and E47 used for introducing BrC6K and Cys/His/Lys, respectively. (b–d) SDS-PAGE (left panels) and high resolution ESI-FTMS (right panels) analyses of affibody mutant proteins Afb-30Uaa-47C (b), Afb-30Uaa-47H (c), and Afb-30Uaa-47K (d). The cross-linking reaction of the Uaa with Cys/His/Lys all result in the loss of HBr. Peaks were observed with monoisotopic masses corresponding to Afb containing the covalent bond formed between the Uaa and the respective target Cys, His, and Lys in each sample. For BrC6K-Cys cross-linked product [M − HBr + H], expected 7772.97 Da, measured 7772.94 Da; [M − Met − HBr + H], expected 7641.93 Da, measured 7641.90 Da. For BrC6K-His cross-linked product [M − Met − HBr + H], expected 7675.98 Da, measured 7675.88 Da. For BrC6K-Lys cross-linked product [M − Met − HBr + H], expected 7667.01 Da, measured 7666.95 Da. da., deamidation; ox., oxidation.

We next tested if BrC6K could cross-link with His in intermolecular PEPC at both pH 7.4 and 8.0. Denatured SDS-PAGE analysis of Afb(D36BrC6K) incubated with MBP-Z(N6H) showed a clear cross-linking band for the protein complex (Supplementary Figure S9). A trace of cross-linking band was observed for the wild-type MBP-Z, which is likely due to Lys4 or Lys7 of wild-type MBP-Z reacting with BrC6K. The intermolecular PEPC efficiency between BrC6K and His is lower than that of intramolecular PEPC, possibly because BrC6K and His are not oriented optimally at the Afb-Z interface for bonding and the affinity between the WT Afb and Z protein is relatively low (6 μM).[18] We expect the efficiency to increase with appropriate site selection at the interface of two proteins possessing high affinity.

The structural flexibility, target diversity, and high cross-linking efficiency of BrC6K may enable us to develop a recombinant stapling technology to staple the secondary structures of proteins and peptides. We set out to staple the α-helix for its broad potential in biological therapeutics.[19−22] Sites 42 and 46, located at the i and i + 4 positions on one α-helix of Afb,[18] were chosen for incorporating Cys and BrC6K, respectively (Figure 3a). The Afb was expressed in *E. coli* and purified by Ni^{2+} affinity chromatography for ESI-FTMS analysis (Figure 3b). Strong peaks were observed with monoisotopic masses clearly corresponding to Afb containing the covalent staple at the introduced sites. Of note, this covalent staple formed completely at native conditions (pH 7.4) in a spontaneous manner without further treatment. The α-helical staple was also successfully generated between BrC6K and Lys in Afb-32Uaa-28K and Afb-45Uaa-49K, albeit requiring incubation at a slightly higher pH (pH 8.8, Supplementary Figures S2b, S10, S11). This strategy will enable the design and recombinant production of various stapled peptides and stapled proteins from cells for developing novel therapeutic biologics.

The efficacy of protein therapeutics is dependent upon the association and dissociation rates between the therapeutic agent and the target.[23−25] Although antibodies can have high affinities for their targets, in many cases such as cancer and infectious diseases it is crucial to completely remove the pathogenic cells

dx.doi.org/10.1021/cb500453a | *ACS Chem. Biol.* 2014, 9, 1956–1961

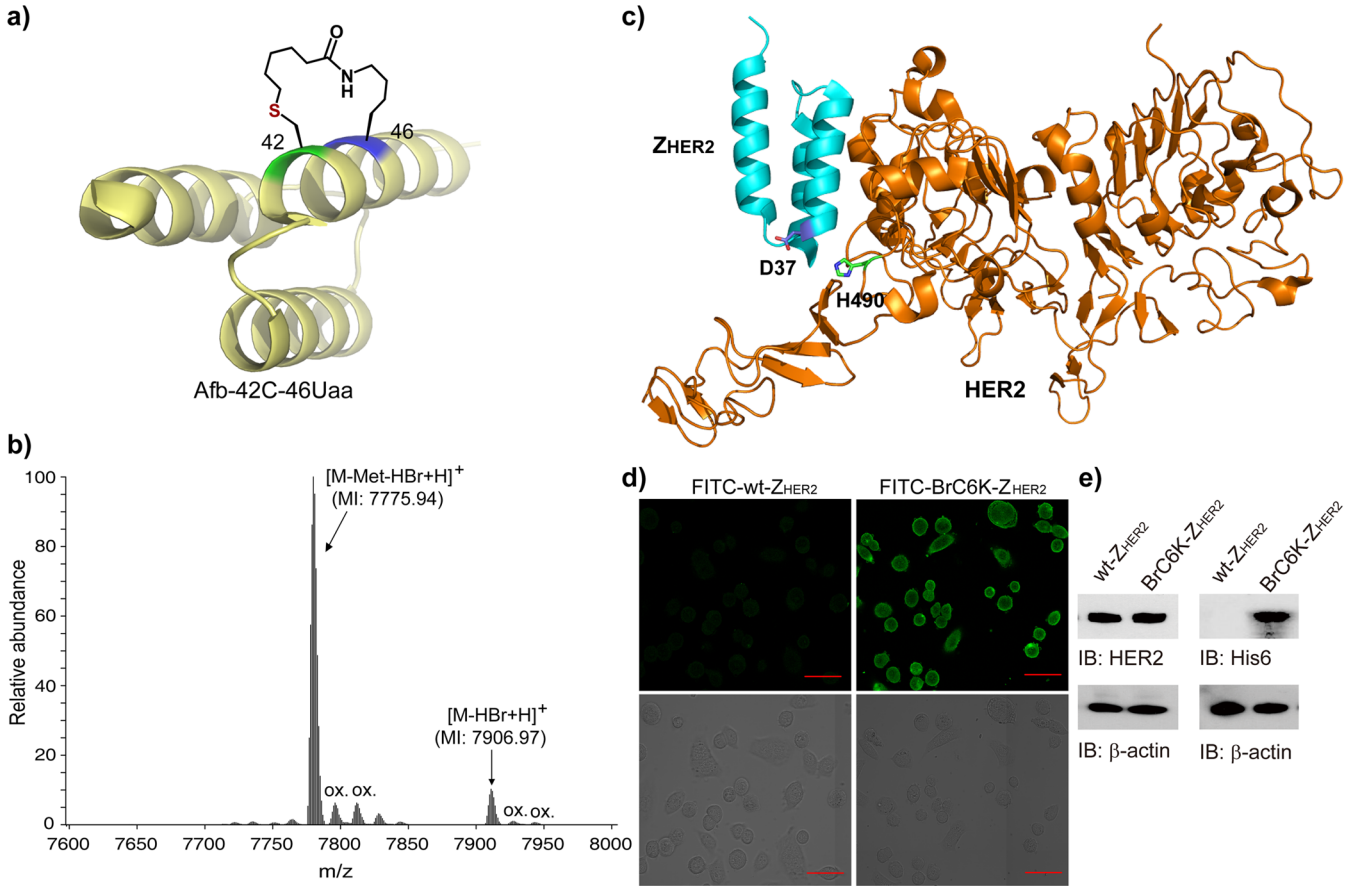


Figure 3. BrC6K enables recombinant stapling of the Afb protein (a,b) and covalent cross-linking of the Z_{HER2} affibody with the endogenous HER2 receptor of breast cancer cells (c–e). (a) Structure of the Afb (PDB ID: 1LP1) showing sites 42 and 46 for introducing Cys and the Uaa BrC6K, respectively, to staple the α-helix. SDS-PAGE analysis of the expressed Afb-42C-46Uaa is shown in Supplementary Figure S2b. (b) High resolution ESI-FTMS analysis of the expressed Afb clearly indicates complete staple formation. Stapled products: [M − HBr + H], expected 7907.01 Da, measured 7906.97 Da; [M − Met − HBr + H], expected 7775.97 Da, measured 7775.94 Da. Nonstapled products: [M + H], expected 7987.93 Da, not detected; [M − Met + H], expected 7856.89 Da, not detected. (c) Crystal structure of Z_{HER2} in complex with the HER2 extracellular domain (PDB ID: 3MZW). Site Asp37 of Z_{HER2} for BrC6K incorporation and the target His490 of HER2 are shown in stick. (d) BrC6K-Z_{HER2}, but not wt-Z_{HER2}, covalently bound to the endogenous HER2 receptor on SKBR3 cells. SKBR3 cells were incubated with the FITC-labeled wt-Z_{HER2} (left) and BrC6K-Z_{HER2} (right) at 37 °C for 3 h, followed by stringent wash and then imaging using identical conditions. Top: confocal FITC fluorescence images. Bottom: bright field image of the same area. Scale bar: 50 μm. (e) Western blot confirms covalent cross-linking of BrC6K-Z_{HER2} with the HER2 receptor. SKBR3 cells were treated with FITC-free wt-Z_{HER2} and BrC6K-Z_{HER2}, respectively, and analyzed using denatured SDS-PAGE followed by Western blotting. A penta-His-specific anbibody was used to detect the His6 tag appended at the C-termini of the wt- and BrC6K-Z_{HER2}.

or the microbial pathogen. Antibody dissociation is also an obstacle in imaging.[23−25] These processes would be significantly enhanced if the therapeutic can covalently cross-link to its target. Here we tested if a covalent affibody could be generated to irreversibly cross-link the endogenous human epidermal growth factor receptor 2 (HER2/ErbB2) on breast cancer cells.[26,27] HER2 plays a central role in oncogenesis; HER2-specific antibodies have been approved for treating breast cancer, and HER2-specific affibodies are in clinical trials.[24,25]

On the basis of the crystal structure[28] of the HER2 extracellular domain in complex with the HER2-specific affibody Z_{HER2}[28−30] (Figure 3c), we decided to incorporate BrC6K into the Asp37 site of Z_{HER2} to target the His490 of HER2. We expressed wt and BrC6K mutant Z_{HER2} proteins and fluorescently labeled them with the FITC dye (Supplementary Figure S12). After incubating these FITC-labeled Z_{HER2} proteins with SKBR3 cells,[31] a HER2-positive breast cancer cell line, both wt-Z_{HER2} and BrC6K-Z_{HER2} bound to the HER2 receptor on cell membrane as shown by the confocal fluorescence microscopy (Supplementary Figure S13). In contrast, no FITC fluorescence was observed from the HER2-negative breast cancer cell line, the MDA-MB-468 cells,[31] after incubation with these Z_{HER2} proteins (Supplementary Figure S14), indicating that the wt- and BrC6K-Z_{HER2} both bound to the HER2 receptor specifically. To explore whether the BrC6K-Z_{HER2} could covalently bind to the HER2 receptor, we incubated the wt- and BrC6K-Z_{HER2}, respectively, with SKBR3 cells and then washed the cells using stringent conditions to disrupt noncovalent binding. Indeed, almost no fluorescence was imaged from SKBR3 cells treated with the wt-Z_{HER2}, whereas bright FITC fluorescence was observed on the membrane of cells treated with the BrC6K-Z_{HER2} (Figure 3d). To further verify covalent cross-linking of the BrC6K-Z_{HER2} with HER2, SKBR3 cells treated with FITC-free wt- and BrC6K-Z_{HER2} were analyzed by SDS-PAGE under denatured conditions followed by immuno-blotting with antibodies specific for HER2 and for the His6 tag appended at the C-terminus of both Z_{HER2} proteins (Figure 3e). A covalent complex of HER2 with BrC6K-Z_{HER2} was detected, but not

dx.doi.org/10.1021/cb500453a | ACS Chem. Biol. 2014, 9, 1956−1961

with wt-Z_{HER2}. Taken together, these results demonstrate that the BrC6K-Z_{HER2} covalently cross-linked with the HER2 receptor of the live breast cancer cell. Irreversible binding to the endogenous membrane receptor has the potential to improve imaging stability and diagnostic sensitivity, and may lead to the development of covalent protein drugs.

In summary, we genetically encoded a versatile electrophilic Uaa into proteins with target diversity and covalent bond-forming flexibility for inter- and intramolecular PEPC. This BrC6K Uaa is capable of reacting with His and Lys in addition to Cys, representing a robust proximity-enabled bioreactive Uaa with targets beyond Cys.[32] An expanding repertoire of natural amino acid residues targetable by proximity-enabled bioreactivity of designed Uaas will significantly broaden the protein diversity and scope of this technology. In addition, by harnessing the structural flexibility and high cross-linking efficiency of BrC6K, we were able to recombinantly staple protein secondary structures and to covalently cross-link native membrane receptors with affibodies in live cells. Recombinant stapling of proteins and covalent targeting of endogenous receptors will enable the design and development of novel biological diagnostics, imaging tools, and therapeutics.

METHODS

Plasmid Construction, Library Construction and Selection, and Uaa Incorporation. These experiments were performed using procedures described previously,[3,6,7] and details are provided in Supporting Information.

Cell Labeling and Imaging. The FITC-labeled wt and BrC6K-containing Z_{HER2} were separately added to SKBR3 and MDA-MB-468 cells (~70% confluent) at the concentration of 300 nM. Cells were cultured at 37 °C with 5% CO_2 in the dark for 3 h, after which the cells were washed using gentle conditions (DPBS buffer, pH 7.5, RT, 10 min, twice) or stringent conditions (500 mM NaCl, 3% Tween 20, 100 mM glycine, pH 3.0, RT, 10 min, twice). The FITC fluorescence was visualized at RT on an Zeiss LSM 710 confocal laser scanning microscope using a 40x objective (excitation 488/30 nm; emission 514/40 nm).

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: lwang@salk.edu.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Innokentiy Maslennikov for NMR measurement. X.-H.C. is partially funded by the Pioneer Fellowship. L.W. acknowledges support from California Institute for Regenerative Medicine (RN1-00577-1) and U.S. National Institutes of Health (1DP2OD004744-01, P30CA014195).

REFERENCES

(1) Xiang, Z., Ren, H., Hu, Y. S., Coin, I., Wei, J., Cang, H., and Wang, L. (2013) Adding an unnatural covalent bond to proteins through proximity-enhanced bioreactivity. *Nat. Methods 10*, 885−888.

(2) Coin, I., Katritch, V., Sun, T., Xiang, Z., Siu, F. Y., Beyermann, M., Stevens, R. C., and Wang, L. (2013) Genetically encoded chemical probes in cells reveal the binding path of urocortin-I to CRF class B GPCR. *Cell 155*, 1258−1269.

(3) Xiang, Z., Lacey, V. K., Ren, H., Xu, J., Burban, D. J., Jennings, P. A., and Wang, L. (2014) Proximity-enabled protein crosslinking through genetically encoding haloalkane unnatural amino acids. *Angew. Chem., Int. Ed. 53*, 2190−2193.

(4) Lins, L., Thomas, A., and Brasseur, R. (2003) Analysis of accessible surface of residues in proteins. *Protein Sci. 12*, 1406−1417.

(5) Kang, H. J., and Baker, E. N. (2011) Intramolecular isopeptide bonds: protein crosslinks built for stress. *Trends Biochem. Sci. 36*, 229−237.

(6) Takimoto, J. K., Dellas, N., Noel, J. P., and Wang, L. (2011) Stereochemical basis for engineered pyrrolysyl-tRNA synthetase and the efficient in vivo incorporation of structurally divergent non-native amino acids. *ACS Chem. Biol. 6*, 733−743.

(7) Lacey, V. K., Louie, G. V., Noel, J. P., and Wang, L. (2013) Expanding the library and substrate diversity of the pyrrolysyl-tRNA synthetase to incorporate unnatural amino acids containing conjugated rings. *ChemBioChem 14*, 2100−2105.

(8) Wang, L., Brock, A., Herberich, B., and Schultz, P. G. (2001) Expanding the genetic code of Escherichia coli. *Science 292*, 498−500.

(9) Wang, Q., Parrish, A. R., and Wang, L. (2009) Expanding the genetic code for biological studies. *Chem. Biol. 16*, 323−336.

(10) Liu, C. C., and Schultz, P. G. (2010) Adding new chemistries to the genetic code. *Annu. Rev. Biochem. 79*, 413−444.

(11) Yanagisawa, T., Ishii, R., Fukunaga, R., Kobayashi, T., Sakamoto, and K. Yokoyama, S. (2008) Multistep engineering of pyrrolysyl-tRNA synthetase to genetically encode N(epsilon)-(o-azidobenzyloxycarbonyl) lysine for site-specific protein modification. *Chem. Biol. 15*, 1187−1197.

(12) Kim, C. H., Kang, M., Kim, H. J., Chatterjee, A., and Schultz, P. G. (2012) Site-specific Incorporation of ε-*N*-crotonyllysine into Histones. *Angew. Chem., Int. Ed. 51*, 7246−7249.

(13) Zhang, M., Lin, S., Song, X., Liu, J., Fu, Y., Ge, X., Fu, X., Chang, Z., and Chen, P. R. (2011) A genetically incorporated crosslinker reveals chaperone cooperation in acid resistance. *Nat. Chem. Biol. 7*, 671−677.

(14) Li, F., Zhang, H., Sun, Y., Pan, Y., Zhou, J., and Wang, J. (2013) Expanding the genetic code for photoclick chemistry in *E. coli*, mammalian cells, and *A. thaliana*. *Angew. Chem., Int. Ed. 52*, 9700−9704.

(15) Lee, Y. J., Wu, B., Raymond, J. E., Zeng, Y., Fang, X., Wooley, K. L., and Liu, W. R. (2013) A genetically encoded acrylamide functionality. *ACS Chem. Biol. 8*, 1664−1670.

(16) Schmidt, M. J., Borbas, J., Drescher, M., and Summerer, D. (2014) A genetically encoded spin label for electron paramagnetic resonance distance measurements. *J. Am. Chem. Soc. 136*, 1238−1241.

(17) Nikic, I., Plass, T., Schraidt, O., Szymanski, J., Briggs, J. A., Schultz, C., and Lemke, E. A. (2014) Minimal tags for rapid dual-color live-cell labeling and super-resolution microscopy. *Angew. Chem., Int. Ed. 53*, 2245−2249.

(18) Hogbom, M., Eklund, M., Nygren, P. A., and Nordlund, P. (2003) Structural basis for recognition by an in vitro evolved affibody. *Proc. Natl. Acad. Sci. U.S.A. 100*, 3191−3196.

(19) Bock, J. E., Gavenonis, J., and Kritzer, J. A. (2013) Getting in shape: controlling peptide bioactivity and bioavailability using conformational constraints. *ACS Chem. Biol. 8*, 488−499.

(20) Bird, G. H., Gavathiotis, E., LaBelle, J. L., Katz, S. G., and Walensky, L. D. (2014) Distinct BimBH3 (BimSAHB) stapled peptides for structural and cellular studies. *ACS Chem. Biol. 21*, 831−837.

(21) Verdine, G. L., and Hilinski, G. J. (2012) Stapled peptides for intracellular drug targets. *Methods Enzymol. 503*, 3−33.

(22) Kritzer, J. A. (2010) Stapled peptides: Magic bullets in nature's arsenal. *Nat. Chem. Biol. 5*, 566−567.

(23) Chan, A. C., and Carter, P. J. (2010) Therapeutic antibodies for autoimmunity and inflammation. *Nat. Rev. Immunol. 10*, 301−316.

(24) Beck, A., Wurch, T., Bailly, C., and Corvaia, N. (2010) Strategies and challenges for the next generation of therapeutic antibodies. *Nat. Rev. Immunol. 10*, 345−352.
(25) Tebbutt, N., Pedersen, M. W., and Johns, T. G. (2013) Targeting the ERBB family in cancer: couples therapy. *Nat. Rev. Cancer 13*, 663−673.
(26) Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000) The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J. 19*, 3159−3167.
(27) Cho, H. S., Mason, K., Ramyar, K. X., Stanley, A. M., Gabelli, S. B., Denney, D. W., and Leahy, D. J. (2003) Structure of the extracellular region of HER2 alone and in complex with the Herceptin Fab. *Nature 421*, 756−760.
(28) Eigenbrot, C., Ultsch, M., Dubnovitsky, A., Abrahmsen, L., and Hard, T. (2010) Structural basis for high-affinity HER2 receptor binding by an engineered protein. *Proc. Natl. Acad. Sci. U.S.A. 107*, 15039−15044.
(29) Orlova, A., Magnusson, M., Eriksson, T. L., Nilsson, M., Larsson, B., Höidén-Guthenberg, I., Widström, C., Carlsson, J., et al. (2006) Tumor imaging using a picomolar affinity HER2 binding affibody molecule. *Cancer Res. 66*, 4339−4348.
(30) Tolmachev, V., Orlova, A., Pehrson, R., Galli, J., Baastrup, B., Andersson, K., Sandström, M., Rosik, D., et al. (2007) Radionuclide therapy of HER2-positive microxenografts using a ^{177}Lu-labeled HER2-specific affibody molecule. *Cancer Res. 67*, 2773−2782.
(31) Holliday, D. L., and Speirs, V. (2011) Choosing the right cell line for breast cancer research. *Breast Cancer Res. 13*, 215−221.
(32) During the preparation of this manuscript, a Uaa targeting lysine for covalent crosslinking of protein−receptor complexes was reported: Furman, J. L., et al. (2014) A genetically encoded aza-Michael acceptor for covalent cross-linking of protein−receptor complexes. *J. Am. Chem. Soc. 136*, 8411−8417.