Programmable polyproteins built using twin peptide superglues

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Programmed connection of amino acids or nucleotides into chains introduced a revolution in control of biological function. Reacting proteins together is more complex because of the number of reactive groups and delicate stability. Here we achieved sequence-programmed irreversible connection of protein units, forming polyprotein teams by sequential amidation and transamidation. SpyTag peptide is engineered to spontaneously form an isopeptide bond with SpyCatcher protein. By engineering the adhesin RrgA from *Streptococcus pneumoniae*, we developed the peptide SnoopTag, which formed a spontaneous isopeptide bond to its protein partner SnoopCatcher with >99% yield and no cross-reaction to SpyTag/SpyCatcher. Solid-phase attachment followed by sequential SpyTag or SnoopTag reaction between building-blocks enabled iterative extension. Linear, branched, and combinatorial polyproteins were synthesized, identifying optimal combinations of ligands against death receptors and growth factor receptors for cancer cell death signal activation. This simple and modular route to programmable “polyproteins” should enable exploration of a new area of biological space.

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Biological events usually depend on the cooperative activity of multiple proteins. Clustering a single kind of protein often greatly enhances biological signals (1), for example in the repeating antigen structures on vaccines (2). However, clustering different kinds of proteins into programmed polyprotein teams (“polyproteins”) is an unmet challenge (3, 4). Protein units can be joined genetically into one long open reading frame, but errors in protein synthesis and misfolding soon become limiting (5, 6). Expressing modules individually and then linking the modules together would overcome these challenges as well as allow independent posttranslational modification of each module. Even the best noncovalent linkages (7–9) or reversible covalent linkages, including disulfide bonds (10, 11), would allow rearrangement of polyproteins, so irreversible covalent linkage is required. There are a limited number of mutually unreactive (orthogonal) chemical reactions (12); therefore, it is impractical to link more than a few building blocks in a one-pot reaction. However, elongating one step at a time allows chain growth using a small number of orthogonal connections (13). If the growing chain is attached to a solid phase, the reacting module can be added in large excess (driving reaction to completion), with unreacted building blocks simply washed away (so separation is unnecessary at each step). Establishing such solid-phase chemistry for connecting amino acids underpinned the breakthroughs in the biological understanding and therapeutic use of peptides (14, 15), whereas the solid-phase synthesis of DNA primers underpinned the revolution in gene amplification and reengineering (16, 17). Solid-phase reaction has also enabled the ligation of peptide fragments to make synthetic proteins (18, 19).

Other important features of a system for synthesizing polyproteins are molecularly defined connections, independence

from any template (20, 21), and simple expression of each module. Nearly quantitative yield for each reaction is required; otherwise, after a few steps the incomplete chains generate hopelessly heterogeneous products. In addition, it is preferable for modules to be modified with peptide tags rather than protein fusion domains (e.g., HaloTag or SNAP-tag) for minimal disruption to module function (22, 23). For unbreakable linkage to a peptide, we previously developed the use of spontaneous isopeptide bond formation (24, 25). SpyTag is a 13-amino-acid peptide that can be genetically fused to the protein of interest, and upon mixing with its protein partner SpyCatcher, an Asp of SpyTag forms a spontaneous isopeptide bond with a Lys of SpyCatcher (26). Reaction occurs with good specificity (27) and under a wide range of conditions, with a connection resistant to boiling in SDS and high force (26).

To enable synthesis of polyproteins, here we first developed a covalent peptide/protein pair (SnoopTag/SnoopCatcher) orthogonal to SpyTag/SpyCatcher. Using iterative Snoop and Spy reactions, we then established the synthesis of linear and branched polyproteins. Polyproteins were analyzed by electrophoresis, chromatography, and mass spectrometry. We then synthesized combinations of polyproteins for sensitive activation of cancer cell death.

Significance

Many biological events depend on proteins working together as a team. Here we establish how to program team formation, covalently linking protein modules step by step. We split a domain from *Streptococcus pneumoniae* to form a peptide and protein pair, SnoopTag and SnoopCatcher, which form an isopeptide bond when mixed together. SnoopTag/SnoopCatcher reacted with each other but not with an alternative peptide/protein pair, SpyTag/SpyCatcher. We formed polyprotein chains by alternating SpyTag reaction with SnoopTag reaction. Cellular signaling often relies on integrated activation of different receptors, so we built polyprotein teams to stimulate Death Receptor and Growth Factor receptors, finding an optimal combination for cell-death induction in cancer cells. Programmable “polyproteins” provide a simple route to investigate or harness biological teamwork.

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Conflict of interest statement: M.H. is an inventor on patent EP2534484, which applies to spontaneous isopeptide bond formation to a peptide tag, and United Kingdom Patent Application No. 1509782.7, which applies to iterative synthesis through isopeptide bonds.

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Results

Design of an Orthogonal Covalent Peptide–Protein Interaction. RrgA is an adhesin from *Streptococcus pneumoniae*, a Gram-positive bacterium that can cause septicemia, pneumonia, and meningitis in humans. A spontaneous isopeptide bond forms in the D4 Ig-like domain of RrgA between residues Lys742 and Asn854 (Fig. 1A) (28). We split the D4 domain in the loop following the N-terminal β -strand and, after exploring various extensions and truncations of the two partners, settled on the peptide tag we termed SnoopTag (residues 734–745) and the protein partner we named SnoopCatcher (residues 749–860; Fig. 1B). To optimize reaction, SnoopCatcher included the mutations G842T, designed to stabilize a β -strand, and D848G, designed to stabilize a hairpin turn close to the reaction site (Fig. S1A). SnoopTag fused to maltose-binding protein (MBP) and SnoopCatcher were expressed efficiently as soluble proteins in the cytosol of *Escherichia coli* and purified by nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography. SnoopTag-MBP and SnoopCatcher, simply upon mixing, formed a complex stable to boiling in SDS (Fig. 1C). Mutations in the putative reactive Lys742 of SnoopTag (SnoopTag KA-MBP) and the putative reactive Asn854 of SnoopCatcher (SnoopCatcher NA) abolished reaction (Fig. 1C). Electrospray ionization mass spectrometry was consistent with the loss of NH_3 resulting from isopeptide bond formation between SnoopCatcher and SnoopTag; acetylated and gluconylated side products common for *E. coli* overexpression were also observed (Fig. 1D). With a 1:1 SnoopCatcher to SnoopTag-MBP,

Fig. 1. Establishing the covalently reactive peptide/protein pair SnoopTag and SnoopCatcher. (A) Spontaneous isopeptide bond formation between Lys and Asn, releasing ammonia. (B) Cartoon of splitting RrgA D4 domain [based on Protein Data Bank (PDB) ID code 2WW8] to make SnoopTag and SnoopCatcher. Reactive residues are in cyan. (C) SnoopTag-MBP reaction with SnoopCatcher, each at 10 μM , after 2 h at 25 $^{\circ}\text{C}$ analyzed by SDS/PAGE with Coomassie staining, alongside controls with Ala mutation of SnoopTag's reactive Lys (KA) or SnoopCatcher's reactive Asn (NA). (D) Isopeptide bond formation between SnoopTag peptide and SnoopCatcher shown by mass spectrometry. (E) Time course of SnoopTag reaction with a 1:1 or 2:1 ratio of SnoopCatcher to SnoopTag-MBP, tested as in C. (F) Time course of SnoopCatcher reaction with a 1:1, 2:1, or 4:1 ratio of SnoopTag-MBP to SnoopCatcher, tested as in C. Error bars are mean $\pm$ 1 SD; $n = 3$. Some error bars are too small to be visible.

Fig. 2. SnoopTag reaction is robust to conditions and orthogonal to SpyTag. (A) For pH dependence, 10 μM SnoopTag-MBP was incubated with 10 μM SnoopCatcher at the indicated pH for 15 min at 25 $^{\circ}\text{C}$ and analyzed by SDS/PAGE with Coomassie staining. (B) Temperature dependence of SnoopTag/SnoopCatcher reaction tested as in A. (C) SnoopTag/SnoopCatcher and SpyTag/SpyCatcher orthogonal reactivity, after incubation for 18 h at 25 $^{\circ}\text{C}$, with each species at 10 μM , determined by SDS/PAGE with Coomassie staining. Error bars are all mean $\pm$ 1 SD; $n = 3$. Some error bars are too small to be visible.

$\sim 80\%$ of SnoopTag-MBP reacted. However, with a twofold excess of SnoopCatcher, SnoopTag-MBP reacted quantitatively (Fig. 1E and Fig. S1B). Similarly with an excess of SnoopTag-MBP, SnoopCatcher was $\sim 100\%$ consumed (Fig. 1F and Fig. S1C). We established that reaction proceeded efficiently from pH 6–9 (Fig. 2A) and 4–37 $^{\circ}\text{C}$ (Fig. 2B). Cysteine is absent from SnoopTag and SnoopCatcher, so, as expected, the reaction was insensitive to DTT (Fig. S1D). No specific buffer component was required, with reaction in PBS as well as in the presence of the detergents Triton X-100 and Tween-20, or high salt (1 M NaCl) (Fig. S1D). The chemical chaperone trimethylamine *N*-oxide (TMAO) (29) modestly enhanced reaction (Fig. S1E).

Spontaneous hydrolysis of an amide bond normally takes years under neutral conditions (30), but we tested if hydrolysis was accelerated in this protein environment. We looked for reversal of the SnoopTag-MBP/SnoopCatcher interaction, by competing with excess of an alternative SnoopTag-linked protein or ammonia, but we did not observe reversibility (Fig. S2).

SnoopTag has a reactive Lys (Fig. 1B), whereas SpyTag has a reactive Asp (26), so we hypothesized that SnoopTag/SnoopCatcher

Fig. 4. Biophysical analysis of polyproteins. (A) Electrospray ionization mass spectrometry to test identity of decamer polyprotein, MBPx-Spy-Catcher:(SnoopTag-Affibody2-SpyTag-SpyCatcher-SnoopCatcher)₄:SnoopTag-Affibody2-SpyTag. Red circles correspond to the decamer, with the charge state of the highest peak marked. (B) Size-exclusion chromatography of the same polyprotein. The *Inset* shows the molecular weight standards.

chromatography of the decamer showed one major peak centered at ~287 kDa, based on calibration with protein standards, consistent with the expected monomeric mass and indicating that there was minimal self-association under these conditions (Fig. 4B). To assess thermostability, the decamer was heated at a range of temperatures and centrifuged to remove aggregates: the decamer remained largely soluble even at 70 °C (Fig. S5A). We also tested decamer integrity to storage: after 1 or 4 d at 25 °C, we saw little degradation and little loss of solubility (Fig. S5B).

Expanding from the initial incorporation of Affibody2 modules into chains, we generated fluorescent protein polyproteins (Fig. S6A). We also generated bottle-brush polyproteins by joining a tandemly linked affibody against HER2, where both the tags were at the N terminus (SnoopTag-SpyTag-Affibody2 × 3), so that the binding units branched from a central core (Fig. S6B) (34).

Application of Polyproteins to Combinatorial Cell Signaling. We established combinatorial synthesis of polyproteins using a simple 96-well plate format. These polyproteins were used to probe the spatial integration between different signaling pathways. Cancer cells are frequently sensitized to cell death induced by Death Receptor signaling, but clinical results have been disappointing using bivalent IgG, which is not potent at cell death induction (35, 36). We set out to explore higher multivalency and how Death Receptor signaling interacts with signaling by growth factor receptors frequently overexpressed in cancer. Therefore, we created combinations of polyproteins with repeats of a nanobody agonist for Death Receptor 5 (DR5) and an affibody to either epidermal growth factor receptor (EGFR), HER2, or type I insulin-like growth factor receptor (IGF1R) (Fig. 5). These proapoptotic polyproteins were synthesized with good purity (Fig. S5A). Each of the combinatorial polyproteins bearing four nanobodies and one affibody unit at each of five positions was tested for effects on viability of the breast cancer cell line MDA-MB-231. Affibody position affected cytotoxicity, with optimal killing by the chain with four nanobodies (N) followed by

an affibody to EGFR (E) (NNNNE chain; Fig. 5B). Negative-control polyproteins containing only affibodies to HER2 (HHHHH), EGFR (EEEE), IGF1R (IIIII), or Taq polymerase (i.e., a ligand not present on the cell surface) (TTTTT) showed no toxicity (Fig. S7). We further assessed the dose dependence of NNNNE cytotoxicity (Fig. 5C). Most of the cytotoxicity of NNNNE was apparent at 24 h (Fig. 5D). NNNNE induced potent activation of caspase signaling, similarly to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL, the endogenous DR5 agonist), and NNNNE's effect was fully blocked by the caspase inhibitor Benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-FMK). HHHHH did not activate caspases (Fig. 5E).

To test the role of valency in polyprotein activation of cell death, we synthesized decamers having varying numbers of anti-DR5 nanobodies alongside either affibodies to EGFR or negative-control affibodies to Taq polymerase (Fig. S7). Polyproteins with one or two nanobodies did not induce cell death, but killing gradually increased as the nanobody valency increased from three to five. With three or four nanobodies in the chain, compared with the anti-Taq control, targeting to EGFR enhanced the polyprotein potency (Fig. S7B).

Discussion

We have generated a modular approach to synthesis of programmed polyproteins, through spontaneous isopeptide bond formation between peptide tags. The polyproteins are linked through irreversible amide bonds and so are stable over time (if protected from proteases) and allow easy analysis by SDS/PAGE. The initiation, extension, and release steps use mild conditions, independent of redox state, and therefore should be applicable to a wide range of proteins. With only a single way for the chain to grow, products are molecularly defined, favoring reproducibility and precise tuning of function. Also, subunits do not need to be connected in an N to C orientation, as we show with bottle-brush polymer architectures. No chemical modification of the module is required, avoiding time-consuming and hard-to-control bioconjugation steps, so our method is accessible to any laboratory able to express recombinant proteins. Combinatorial synthesis of polyproteins allows rapid testing of patterns able to give potent cellular signal activation.

Spontaneous isopeptide bond formation has the advantage of a simple reaction pathway between two functional groups having low intrinsic reactivity (an amine with a carboxylic acid or a carboxamide), so there is little side reaction (26, 37) and yields for each step were almost quantitative (38). This enabled a high purity of polyproteins after nine consecutive reactions. Polyprotein assembly is not traceless, leaving a SpyCatcher-SnoopCatcher unit between each module. In future work, it will be interesting to explore SpyCatcher-SnoopCatcher units with alternative sizes and orientations, as well as using other linkage chemistries for solid-phase synthesis. Sortase, subtiligase, transglutaminase, and split inteins enable covalent protein-protein interaction that is either traceless or leaves a short peptide tag (39–43), and these approaches will be valuable to test in the future with MBPx attachment and maltose elution. However, those reactions pass through a (thio)ester intermediate, so hydrolysis may compete with ligation (39, 40). Artificial amino acids for bio-orthogonal reaction would enable minimal modification but with some issues: the increased complexity of module expression and competing reactions [such as azide reduction (44), alkyne reaction with thiols (45), and spontaneous tetrazine degradation (46)] as well as competition from suppression of stop codons by normal amino acids (47). Recent work demonstrated the synthesis of ubiquitin homo-polymers by azide-alkyne (48), thiol-ene (49), or native chemical ligation reactions (50). A potential disadvantage of our approach is that Tag/Catchers are not human and therefore are likely to be immunogenic, although nonhuman connectors are advantageous for vaccination. Also, each module must have a single SpyTag and SnoopTag, which is straightforward for monomers or

Fig. 5. Combinatorial synthesis of polyproteins. (A) Chain synthesis with four anti-DR5 nanobodies and one affibody-based unit at various positions, analyzed by SDS/PAGE with Coomassie staining. (B) Polyprotein effect on viability of MDA-MB-231 cells after 40 h of incubation with each chain from A at 200 ng/mL, shown as a heat-map. (C) Viability of MDA-MB-231 cells at 40 h with titration of NNNNE and HHHHH control (N, anti-DR5 nanobody; E, AffiEGFR; H, AffiHER2 × 3). (D) Time course of cytotoxicity with 200 ng/mL NNNNE on MDA-MB-231 cells. (E) Caspase reporter activation in response to NNNNE, KillerTRAIL (a DR5 agonist) or HHHHH with or without Z-VAD-FMK pan-caspase inhibitor. (Error bars in each case are mean ± 1 SD; $n = 3$. Some error bars are too small to be visible.)

heteromultimers but needs careful attention for homo-multimers. As one makes larger polyproteins, their solubility and colloidal characteristics will change, just as multidomain extracellular matrix proteins such as fibronectin cannot be treated in the same way as small globular proteins. However, the affibody polyprotein showed good stability over time and to heating. Polyproteins should be a powerful platform to dissect the spatial requirements for cellular signaling, such as in immunity and differentiation (51–53). Other applications of this simple route to new biological architectures may include vaccination (2), biomaterials (34, 54–56), multienzyme organization (9), and enhancing capture of circulating tumor cells (57).

Materials and Methods

Isopeptide Bond Reconstitution Reactions. *SI Materials and Methods* provides a full description of cloning, protein expression and purification, MS, size-exclusion chromatography, cell culture, stability testing of chains, combinatorial assembly of chains, and caspase activation assay. To assess the formation of a covalent bond between SnoopTag and SnoopCatcher, proteins were mixed each at 10 μ M final concentration in Tris-buffered saline (TBS) pH 8.0 containing 1.5 M TMAO (Sigma-Aldrich). Reactions were stopped by adding 6× SDS loading buffer [0.23 M Tris-HCl, pH 6.8, 24% (vol/vol) glycerol, 120 μ M bromophenol blue, 0.23 M SDS]. Samples were subsequently heated using a Bio-Rad C1000 thermal cycler at 95 °C for 5 min, before SDS/PAGE on 16% (wt/vol) polyacrylamide gels using an XCell SureLock gel container (Life Technologies) at 200 V. SDS/PAGE running buffer was Tris-glycine, except Tris-acetate buffer was used to improve resolution of high-molecular-weight products, as described previously (7). Gels were stained with InstantBlue Coomassie stain (Triple Red Ltd.), and bands were densitometrically analyzed using a Gel Doc XR imager and Image Lab 3.0 software (Bio-Rad).

Solid-Phase Synthesis of Chains. We applied 40 μ L slurry amylose resin (New England BioLabs) to a 1 mL poly-prep column (Bio-Rad), rinsed the resin with 1 mL MilliQ water, and equilibrated the resin with 1 mL TBS pH 8.0. We added 320 pmol MBP-SpyCatcher in TBS pH 8.0 in a final volume of 80 μ L to

the resin and incubated the resin at 25 °C for 1 h with 700 rpm shaking on a ThermoMixer comfort (Eppendorf). Unreacted protein was removed by gravity flow, and resin was washed with 1 mL wash buffer (50 mM Tris-HCl pH 8.0 with 500 mM NaCl). We added 3 nmol monomer containing a SnoopTag and a SpyTag in TBS pH 8.0 in a final volume of 80 μ L to the resin and incubated the resin at 25 °C for 1 h with 700 rpm shaking. Unreacted SnoopTag/SpyTag monomer was removed from the column by gravity flow and resin washed with 1 mL wash buffer. We added 4 nmol SpyCatcher-SnoopCatcher in TBS pH 8.0 with 1.5 M TMAO to the resin and incubated the resin at 25 °C for 2 h with 700 rpm shaking. Unreacted SpyCatcher-SnoopCatcher was removed by gravity flow, and resin was washed with 1 mL wash buffer. Chains were produced by sequential addition of SnoopTag/SpyTag monomer and SpyCatcher-SnoopCatcher, according to the conditions described above. Chains were eluted, after resin washing, by applying onto the column 40 μ L TBS pH 8.0 containing 50 mM D-maltose (Sigma-Aldrich) and incubating the resin at 25 °C for 10 min with 700 rpm shaking. Chains were collected by centrifuging the column in a 1.5 mL micro-centrifuge tube for 10 s at 17,000 × g . For SDS/PAGE testing after each step, samples were eluted as previously described, mixed with 6× SDS loading buffer, and heated at 95 °C for 5 min. SDS/PAGE was performed on 10% (wt/vol) and 4% (wt/vol) Tris-acetate gels at 150 V.

For biotin-SpyCatcher-based assembly, 40 μ L of slurry monomeric avidin resin (Thermo Scientific) was applied to a 1 mL polyprep column, rinsed with 1 mL MilliQ water, and equilibrated with 1 mL TBS pH 8.0. Biotin-SpyCatcher in TBS pH 8.0 in a final volume of 80 μ L at 4 μ M was added to the resin and incubated at 25 °C for 1 h with 700 rpm shaking. Unreacted biotin-SpyCatcher was removed by gravity flow, resin was washed with 1 mL wash buffer, and sequential addition of SnoopTag-AffiHER2-SpyTag and SpyCatcher-SnoopCatcher was performed as described above. After resin washing, chains were eluted by applying 40 μ L of 1 mM D-biotin in TBS pH 8.0 and incubating the solution at 25 °C for 4 h with 700 rpm shaking. Chains were collected as previously indicated and analyzed by SDS/PAGE on 16% (wt/vol) and 8% (wt/vol) Tris-glycine gels with Coomassie staining.

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