Novel method could improve the performance of proteins used therapeutically

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Whitehead Institute scientists have created a method that site-specifically modifies proteins to exert control over their properties when administered therapeutically. The technique should be useful to increase potency, slow metabolism, and improve thermal stability of therapeutically useful proteins, such as interferon alpha 2 (IFN-alpha 2), which is used to treat variety of diseases, including leukemia, melanoma, and chronic hepatitis C.

The method, reported this month in Proceedings of the National Academy of Sciences (*PNAS*), uses the enzyme sortase A and can be applied to tailor proteins that possess a structure found in IFN-alpha 2, referred to as a four-helix bundle. Such proteins include erythropoietin (EPO), granulocyte colony-stimulating factor 3 (GCSF-3, known as filgrastim and marketed as Neupogen®), interleukin (IL) 2 (known as aldesleukin and marketed as Proleukin), IL-4, IL-7, IL9-, and IL-15.

"In the course of this work, the first author of the *PNAS* paper, Maximilian Popp, together with other members of the lab, has put together a nice palette of sortase-based techniques that now allow us to modify a large variety of different proteins, and equip them with properties and behaviors that cannot be easily specified by more standard molecular biological techniques," says Whitehead Member Hidde Ploegh. "I see the value of these approaches first and foremost in their general applicability and ease of use."

IFN-alpha 2 is a cytokine, a hormone-like substance that usually acts on

cells other than those that produce the protein. Upon binding the cytokine, the recipient cell responds, for example by starting to divide and proliferate, or by exercising certain functions of benefit to the organism. Like other cytokines used for therapeutic purposes, IFN-alpha 2 can be a finicky drug. It is thermally unstable and must be continuously refrigerated to maintain its potency, a requirement that limits IFN-alpha 2's use in areas with intermittent or no electricity. Also, IFN-alpha 2's relatively short half-life (and resulting rapid clearance from the body) often necessitates frequent injections when the drug is used to treat certain conditions.

To keep therapeutic IFN-alpha 2 active in the body longer, the current strategy is to tack long polyethylene glycol (PEG) chains onto the protein to turn them into effective drugs. This so-called PEGylation not only masks IFN-alpha 2 from the patient's immune system but also increases the time the body needs to break it down. However, because current approaches to PEGylation are not specific, the PEG chains can block or alter the protein's normal binding site—an unintended consequence of this modification that can diminish IFN-alpha 2's potency by as much as 90%.

Seeking greater precision in attaching PEG chains, Popp, who is a graduate student in the Ploegh lab, used the enzyme sortase A to cleave IFN-alpha 2 at a specific site on the protein, engineered so that it would be recognized by the sortase. Then, a small molecule bearing the PEG chain was attached at the site cleaved by sortase. When Popp tested for biological activity, the resulting IFN-alpha 2 was highly potent, indicating that the PEG chains were not interfering with the drug's binding ability.

Popp also used sortase A to suture PEG chains to the cytokine GCSF-3. When he tested the PEGylated version in mice, it remained in the bloodstream significantly longer and evoked a more robust and

prolonged response than a non-PEGylated version. By using sortase A's inherent precision to attach PEG chains, Popp could replace the less precise chemistry-based technique with a highly effective method that should have broader applications.

Next, Popp addressed IFN-alpha 2's thermal stability. Previously, the Ploegh lab stabilized linear polypeptides like IFN-alpha 2 by molecularly gluing their ends together to form circles. A few such cyclic proteins are found in nature. Once circularized, cyclic proteins are often more stable than their linear precursors. This forced looping can interfere with the function of some cell-signaling proteins, but because IFN-alpha 2's binding site is not near its ends, the function of IFN-alpha 2 is unaffected when its ends are joined to form a circle.

To create a cyclic version of IFN-alpha 2, Popp used sortase A to join the two ends of IFN-alpha 2. When he heated the cyclic form of IFN-alpha 2, it was more resistant to breakdown than its linear counterpart and remained biologically potent even after boiling. Popp then tested the circular, PEGylated version and the linear version in mice. The modified version was metabolized more slowly than the linear version and maintained its thermal stability, demonstrating that this simple technique can significantly enhance desirable properties of a therapeutically relevant protein without sacrificing its potency.

"We really take advantage of the site specificity of the sortase enzyme. Placing a molecular suture like that can't really be done by other means. So I think this method is of value to the protein engineering field in general," says Popp. "The reaction itself is easy, but it took some time to actually figure out how to do these transformations. Once we figured that out, the technique was robust and reproducible."

More information: "Sortase-catalyzed transformations that improve the properties of cytokines" *PNAS*, online February 9, 2011.

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