Protein Loop Grafting to Construct a Variant of Tissue-type Plasminogen Activator That Binds Platelet Integrin $\alpha_{IIb}\beta_3$*

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Protein-protein interactions can be guided by contacts between surface loops within proteins. We therefore investigated the hypothesis that novel protein-protein interactions could be created using a strategy of "loop grafting" in which the amino acid sequence of a biologically active, flexible loop on one protein is used to replace a surface loop present on an unrelated protein. To test this hypothesis we replaced a surface loop within an epidermal growth factor module with the complementarity-determining region of a monoclonal antibody. Specifically, the HCDR3 from Fab-9, an antibody selected to bind the $\beta_3$-integrins with nanomolar affinity (Smith, J. W., Hu, D., Satterthwait, A., Pinz-Sweeney, S., and Barbasis, C. F., III (1994) J. Biol. Chem. 269, 32788-32795), was grafted into the epidermal growth factor-like module of human tissue-type plasminogen activator (t-PA). The resulting variant of t-PA bound to the platelet integrin $\alpha_{IIb}\beta_3$ with nanomolar affinity, retained full enzymatic activity, and was stimulated normally by the physiological co-factor fibrin. Binding of the novel variant of t-PA to integrin $\alpha_{IIb}\beta_3$ was dependent on the presence of divalent cations and was inhibited by an RGD-containing peptide, demonstrating that, like the donor antibody, the novel t-PA binds specifically to the ligand-binding site of the integrin. These findings suggest that surface loops within protein modules can, at least in some cases, be interchangeable and that phage display can be combined with loop grafting to direct proteins, at high affinity, to selected targets. In principle, these targets could include not only other proteins but also peptides, nucleic acids, carbohydrates, lipids, or even uncharacterized markers of specific cell types, tissues, or viruses.

Development of the ability to create novel protein-protein interactions promises to provide important new therapeutic agents as well as unique tools and reagents for the study of key biological processes. Such advances in protein engineering may also provide seminal information about how proteins interact.

One strategy for manipulating protein-protein interactions is to employ random or nearly random (e.g. alanine scanning) site-directed mutagenesis to identify amino acid residues critical for binding affinity and specificity. Cumbersome, large scale mutagenesis efforts followed by laborious, time-consuming assays of individual, mutated proteins can sometimes extend this approach to create new molecular interactions. For example, every residue in human growth hormone was scanned for activity prior to re-engineering the molecule to bind the prolactin receptor (2), and a similar strategy facilitated the construction of a variant of interleukin-3 that binds the monoclonic $\alpha_3$ receptor with higher affinity than it binds the interleukin-3 $\alpha_3\beta_3$ receptor (3). An important current challenge, therefore, is to create more rapid and efficient strategies to engineer proteins with new binding properties. A recent approach that can obviate the need to perform large numbers of binding assays with individual, mutated proteins is biopanning using phage display systems. Although originally conceived as a means of screening vast numbers of peptide motifs (4), it is now apparent that whole proteins and domains within proteins can be manipulated using the phage selection strategy (5).

Another strategy that has been used to modify protein-protein interactions is the addition, deletion, or substitution of entire domains within proteins (6-12). Although it is often successful, this strategy provides an extremely low resolution picture of protein-protein interactions; consequently, manipulation of entire domains is often followed by the construction and analysis of numerous point mutants as described above. Severe limitations also arise if a protein domain of interest carries more than one important biological activity; maintaining one activity (e.g. functionally significant domain-domain interactions) while altering another (e.g. high affinity binding to a co-factor or receptor) can be problematic. An approach that might overcome both of these limitations would be to transfer between proteins not entire domains but rather defined structural elements within protein domains. We therefore focused in this study on grafting flexible protein loop structures, which often guide protein binding phenomena (13). Flexible loops are found on the surface of most protein modules and exist as short stretches of amino acids that connect regions of defined secondary structure. Although crystallographic and NMR studies show that these loops are usually less well defined than helices and $\beta$-sheets, their conformational freedom is normally restricted substantially compared with free peptides. Consequently, the binding activities of surface loops in proteins usually differ significantly from those of the corresponding linear amino acid sequence (14).

To substantially increase the speed and efficiency by which protein engineering can be used to confer novel binding activities to a selected protein, we adopted a strategy that combined phage display and loop grafting. To test this strategy, we replaced amino acids in a surface loop within the epidermal growth factor (EGF) domain of tissue-type plasminogen activator with residues from the CDR3 of Fab-9. The resulting new t-PA bound to the platelet integrin $\alpha_{IIb}\beta_3$ with nanomolar affinity, retained full enzymatic activity, and was stimulated normally by the physiological co-factor fibrin. Binding of the novel t-PA to integrin $\alpha_{IIb}\beta_3$ was dependent on the presence of divalent cations and was inhibited by an RGD-containing peptide, demonstrating that, like the donor antibody, the novel t-PA binds specifically to the ligand-binding site of the integrin. These findings suggest that surface loops within protein modules can, at least in some cases, be interchangeable and that phage display can be combined with loop grafting to direct proteins, at high affinity, to selected targets. In principle, these targets could include not only other proteins but also peptides, nucleic acids, carbohydrates, lipids, or even uncharacterized markers of specific cell types, tissues, or viruses.

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plasminogen was varied from 0.0125 to 0.2 in the presence of the peptide Gly-Pro-Arg-Pro. The concentration of Lys-plasminogen (American Diagnostica) and Spectrozyme PL were present in a total volume of 100 μl. Assays were performed either in the presence of buffer, 25 μg/ml DESAFIB, 100 μM cyanogen bromide fragments of fibrinogen (American Diagnostica), or 100 μM fibrinogen. Assays were performed in microtiter plates, and the optical density at 405 nm was read every 30 s for 1 h in a Molecular Dynamics Thermomax.

RESULTS

The Use of an Antibody CDR as a Donor and an EGF Module as a Recipient in Loop Grafting—The objective of this study was to test the concept that biologically active surface loops, at least in some cases, can be grafted between proteins of different backbone structure. As our donor we chose a CDR from Fab-9, a human antibody that we recently engineered to bind the ligand-binding pocket of the β3-integrins (27). The HCDR3 loop from this antibody contains the active sequence SFGRGDIRN and is bounded by two cysteine residues. While Fab-9 binds to β3-integrins with nanomolar affinity, synthetic peptides with the HCDR3 sequence of Fab-9 display at least 100-fold lower affinity for integrin (14). The criteria for a successful grafting of the loop, therefore, is the retention of high affinity for integrin. An EGF module was chosen as a recipient because the structure of several proteins containing this module is available and shows that EGF contains a large, exposed loop that contains a β-turn structure. This loop is bounded by two disulfide pairs (28). Using oligonucleotide-directed site-specific mutagenesis and conventional cloning techniques, we constructed a cDNA encoding a variant of t-PA in which residues forming the exposed loop on the surface of the EGF domain have been replaced by amino acids found in the active loop of Fab-9 (Fig. 1). The Arg of the RGD motif was placed at residue 66 of t-PA, which, based upon NMR structures of EGF modules (28), is near the apex of this disulfide-bounded loop. Because the CDR sequence of Fab-9 is 1 residue shorter than the EGF loop, we maintained the carboxyl-terminal residue within the EGF loop as Val, the native residue at this position in t-PA. The new variant of t-PA is referred to as LG-t-PA.

The Modified t-PA Maintains Full Enzymatic Activity and Is Stimulated by the Physiological Co-factor Fibrin—a kinetic analysis of the activity of wild type and LG-t-PA toward the small chromogenic substrate Spect-PA is summarized in Table I.
The close correspondence between values of $K_m$, $k_{cat}$, and $k_{cat}/K_m$ for the two enzymes in this assay clearly demonstrates that LG-t-PA maintains full activity toward synthetic substrates.

Table II presents the results of a kinetic assay of plasminogen activation in the presence of the co-factor fibrin by t-PA and LG-t-PA. The kinetic constants of LG-t-PA for plasminogen activation are very similar to those of wild type t-PA; $k_{cat}/K_m$ values for the two enzymes vary by approximately 10% in this assay. LG-t-PA, therefore, maintained full enzymatic activity not only toward small synthetic substrates but also toward the natural protein substrate plasminogen.

The activity of wild type t-PA is stimulated by fibrin, fibrinogen, and cyanogen bromide fragments of fibrinogen, and we and others have reported that mutations mapping to at least five distinct regions of the enzyme can differentially affect stimulation of t-PA by these distinct co-factors (29, 30). To examine whether mutations present in LG-t-PA affected stimulation of the enzyme by any of these three co-factors, we performed standard indirect chromogenic assays of the two enzymes in the presence of each of the co-factors. The results of these assays are depicted in Fig. 2 and indicate that the mutations present in LG-t-PA have not significantly compromised interaction of the enzyme with any of the co-factors.

The role, if any, of the EGF domain of t-PA in mediating stimulation of the enzyme by fibrin has generated controversy and conflicting reports (9, 12, 31). Although this study will not resolve the issue, our results do argue strongly that residues 63–71, which form part of an antiparallel $\beta$-sheet and a $\beta$-turn on surface of the EGF domain, do not play a role in stimulation of the enzyme by fibrin or fibrinogen.

LG-t-PA Binds to $\beta_3$-Integrins with Nanomolar Affinity—Two integrins containing the $\beta_3$ subunit have been described. These are platelet integrin $\alpha_{IIb}\beta_3$ and integrin $\alpha_d\beta_3$. Both of these integrins bind the antibody Fab-9 with nanomolar affinity (14, 27). The ability of the modified t-PA, LG-t-PA, to bind the two $\beta_3$-integrins was tested using purified integrin and culture supernatant from cells transfected with the cDNA encoding LG-t-PA. An indirect binding assay was used to measure binding affinity of the LG-t-PA for integrins because purified t-PA cannot be maintained at high concentrations in buffers that are compatible with integrin binding assays.2

FIG. 1. A representation of the NMR structure of murine epidermal growth factor adapted from Kohda et al. (28). Residue 23 of the murine protein corresponds to residue 65 of human t-PA. The $\beta$-turn formed by residues 23–28 of the murine protein may be extended in t-PA due to the occurrence of a three-amino acid insertion, $^{67}$YFS$^{69}$, at the location indicated by the large arrow. The primary sequence of the substituted region of LG-t-PA and the corresponding region of wild type t-PA are indicated at the bottom of the figure. Dashes in the LG-t-PA sequence indicate identity to the wild type enzyme.

The table presents the kinetic constants of LG-t-PA for plasminogen activation.

### Table I: Kinetic constants for cleavage of the chromogenic substrate Spec t-PA

| Enzyme  | $k_{cat}$ | $K_m$  | $k_{cat}/K_m$ |
|---------|-----------|--------|--------------|
| t-PA    | 44        | 0.3    | $1.5 \times 10^5$ |
| LG-t-PA | 45        | 0.3    | $1.5 \times 10^5$ |

The values for $K_m$, $k_{cat}$, and $k_{cat}/K_m$ are in units of $\mu M$, $s^{-1}$, and $s^{-1} \mu M^{-1}$, respectively.

### Table II: Activation of plasminogen in the presence of fibrin

| Enzyme  | $k_{cat}$ | $K_m$  | $k_{cat}/K_m$ |
|---------|-----------|--------|--------------|
| t-PA    | 0.10      | 0.02   | $5.0 \times 10^5$ |
| LG-t-PA | 0.09      | 0.02   | $4.5 \times 10^5$ |

The values for $K_m$, $k_{cat}$, and $k_{cat}/K_m$ are in units of $\mu M$, $s^{-1}$, and $s^{-1} \mu M^{-1}$, respectively.

The diagrams and figures are as follows:

**FIG. 2.** Standard indirect chromogenic assay of plasminogen activation by wild type (WT) and LG-t-PA in the presence of buffer (●), fibrin monomers (○), fibrinogen (□), or cyanogen bromide fragments of fibrinogen (▲).

2 J. W. Smith, K. Tachias, and E. L. Madison, unpublished observations.
sequently, t-PA binding to integrin was measured by determining the amount of t-PA present in conditioned media from transfected COS cells and using this media as a source of t-PA. t-PA that bound to purified integrin was reported as t-PA activity and was measured using a standard indirect chromogenic assay. The binding isotherm shown in Fig. 3 shows that the LG-t-PA binds purified integrin \( \alpha_{IIb}\beta_3 \). Nonspecific binding was assessed by including EDTA in the binding study because ligand binding to integrins is dependent upon divalent cations (23, 32). The binding of LG-t-PA to \( \alpha_{IIb}\beta_3 \) is specific and saturable, exhibiting a \( K_D \) of approximately 0.9 nM. This \( K_D \) is the average of four similar experiments in which the dissociation constant ranged from 0.5 to 1.3 nM. In the experiment shown in Fig. 3, the apparent \( K_D \) is 0.5 nM. The average \( K_D \) for this interaction compares favorably with the \( K_D \) of Fab-9 for this integrin of 5 nM (14). Like the protein containing the parent loop, Fab-9, the modified t-PA also bound to integrin \( \alpha_{IIb}\beta_3 \) (data not shown). The affinity of the modified t-PA for this integrin was also high, with an apparent \( K_D \) of 1.8 nM, similar to the \( K_D \) of 1.7 nM exhibited by Fab-9. Thus, by contrast to linear and cyclic synthetic peptides with sequences that are identical to the HCDR3 of Fab-9, LG-t-PA maintained the high affinity for integrins exhibited by Fab-9.

Loop-grafted t-PA Binds to the Ligand-binding Site of \( \beta_3 \)-Integrins—One of the hallmarks of ligand binding to the \( \beta_3 \)-integrins is that small synthetic peptides with the RGD sequence can block their binding. The ability of RGD peptides to block LG-t-PA was tested by a simple competition assay. A concentration range of RGD peptide was included during the binding reaction with the modified t-PA. As a control, a synthetic peptide with the same composition but random sequence was also used as competitor. Bound t-PA was detected with the indirect activity assay described under “Materials and Methods.” As shown in Fig. 4, the peptide with sequence GRGDSP blocked the binding of LG-t-PA to integrin, but a peptide with a random sequence had no effect on binding. The data shown are for integrin \( \alpha_{IIb}\beta_3 \) and nearly identical data were obtained when the RGD peptide was used to block binding of LG-t-PA to purified integrin \( \alpha_{IIb}\beta_3 \) (data not shown). These data show that, like Fab-9, LG-t-PA binds to the ligand-binding site of the \( \beta_3 \)-integrins.

**DISCUSSION**

The major finding of this study was that amino acids forming a biologically active, flexible surface loop on one protein could be grafted into another, unrelated protein and still maintain their initial binding activity. The protein sequence of the grafted loop was originally optimized by mutagenesis and affinity maturation of the donor protein, monoclonal antibody Fab-9, using phage display. To our knowledge these results provide the first example in which a flexible protein loop, optimized by phage display, has been inserted into another...
protein backbone to successfully endow the recipient protein with novel, high affinity binding properties.

This study differs significantly both in approach, and in end results, from previously reported studies where RGD sequences have been inserted into non-adhesive proteins (33–35). Although these efforts have successfully targeted recipient proteins to integrins, substantial quantitation of binding affinities has not been reported for these proteins nor has the integrin target been identified. In these studies an RGD sequence was inserted into either lysozyme or calpastatin, and the apparent target plasminogen activator to blood clots by conjugating the resulting variant of t-PA merits consideration as an improved thrombolytic agent for the treatment of acute myocardial infarction and other thromboembolic disorders. Efforts to target plasminogen activators to blood clots by conjugating the enzyme to antibodies or Fab fragments of antibodies directed against either fibrin (36, 37) or platelet integrins (38) have been previously reported and have often demonstrated that this strategy can enhance the thrombolytic potency of the plasminogen activator both in vitro and in vivo. For example, chemical conjugation of monodonal antibody 7E3 (anti-IIb-IIIa) to urokinase yielded a molecule that was 25-fold more potent in lysing platelet-rich clots than wild type urokinase (38). LG-t-PA, therefore, due to its novel binding properties, may also exhibit enhanced potency toward the platelet-rich arterial thrombi that precipitate acute myocardial infarction.

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