Random PCR mutagenesis screening of secreted proteins by direct expression in mammalian cells

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ABSTRACT We have developed a general method for screening randomly mutagenized expression libraries in mammalian cells by using fluorescence-activated cell sorting (FACS). The cDNA sequence of a secreted protein is randomly mutagenized by PCR under conditions of reduced Taq polymerase fidelity. The mutated DNA is inserted into an expression vector encoding the membrane glycosphospholipid anchor sequence of decay-accelerating factor (DAF) fused to the C terminus of the secreted protein. This results in expression of the protein on the cell surface in transiently transfected mammalian cells, which can then be screened by FACS. This method was used to isolate mutants in the kringle 1 (K1) domain of tissue plasminogen activator (t-PA) that would no longer be recognized by a specific monoclonal antibody (mAb387) that inhibits binding of t-PA to its clearance receptor. DNA sequence analysis of the mutants and localization of the mutated residues on a three-dimensional model of the K1 domain identified three key discontinuous amino acid residues that are essential for mAb387 binding. Mutants with changes in any of these three residues were found to have reduced binding to the t-PA receptor on human hepatoma HepG2 cells but to retain full clot lysis activity.

Random mutagenesis of a DNA region of interest coupled with a screening system is a powerful method for the study of a gene or its regulatory functions (1, 2). A saturation mutagenesis technique has been developed for expression of mutated surface antigens on the surface of transfected mammalian cells (3, 4). The population of cells expressing mutated antigens that would no longer bind to specific monoclonal antibodies (mAbs) was selected by specific complement-mediated lysis of cells that retained the epitope of interest. This technique works only for screening mutated proteins that are normally expressed on the cell surface. It would therefore be useful to develop a screening method for selection of mutagenized proteins that are normally secreted by cells, as many proteins of therapeutic interest are secretory proteins. This paper reports the development of a cloning vector for expression of secretory proteins as fusion proteins on the cell surface of transfected mammalian cells. The secreted protein is displayed on the cell surface by fusion with the glycosphospholipid membrane anchor of decay-accelerating factor (DAF). Tissue-type plasminogen activator (ref. 5; t-PA), which is normally secreted, is used as a model protein. PCR mutagenesis (6) is used to generate random mutations within the kringle 1 (K1) domain of t-PA. Fluorescence-activated cell sorting (FACS) is used to screen for t-PA mutants possessing loss of an epitope to a specific mAb, whose nonlinear binding domains overlap with the t-PA clearance receptor contact regions.

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MATERIALS AND METHODS

Plasmid Construction. The t-PA–DAF fusion protein expression vector construct was made by inserting synthetic DNA oligonucleotides encoding the last 37 amino acids of DAF (7) behind the last codon of t-PA. The resulting DNA fragment encoding the t-PA–DAF fusion protein was then cloned into the polylinker region of pRK.CXHRN (8) in such a way that expression of the fusion protein is directed by the cytomegalovirus promoter. This plasmid, pRK-t-PA-DAF, was used as the starting material for all mutagenesis experiments.

Mutagenesis. Site-directed mutagenesis (9) was used to engineer two restriction sites (Cla I at amino acids 86–87 and Nhe I at amino acids 199–200) into the coding region of t-PA without changing the amino acid coding sequence of t-PA. Specific PCR primers were then used to amplify the DNA region spanning the Cla I and Nhe I sites using conditions of reduced Taq polymerase fidelity (6). The resulting mutagenized PCR products were digested with Cla I and Nhe I and cloned into a pRK-t-PA-DAF.

DNA Sequencing. Plasmid DNA isolated from selected mutants was sequenced (10) with a synthetic primer that would hybridize to the coding strand of t-PA corresponding to amino acids 213–218.

Cell Culture. The human embryonic kidney cell line TSA201, a 293 cell line derivative that expresses simian virus 40 large tumor antigen, and the human hepatoma HepG2 were cultured in F-12/Dulbecco’s modified Eagle’s medium (DMEM) (50:50) plus 10% fetal calf serum and antibiotics.

Transfection and Selection. TSA201 cells (3 × 10⁵ cells per ml) were electroporated with the mutagenized libraries (4 μg). The transfection efficiency under these conditions was ~10%, which increased the percentage of single copy transfections (11). Forty-eight hours after transfection, the cells were removed from plates with 0.04% EDTA and stained with two antibodies recognizing (i) an epitope within the mutagenized K1 domain (mAb387) and (ii) an epitope within the protease domain (mAb372). Cells, negative for i and positive for ii, were sorted. Plasmid recovery was essentially as described by Hirt (12). Bacteria transformation was by electroporation (12). After two rounds of transient selection, individual bacteria colonies were picked, and miniscreeen DNA was isolated and transfected separately into TSA201 cells by calcium phosphate transfection (13). Staining for the two epitopes was assessed by FACS 48 hr later. Isolated epitope loss mutants were subcloned into a wild-type t-PA expression vector, pRK-t-PA, substituting the mutagenized K1 cassette for that of the wild type. The mutant

Abbreviations: mAb, monoclonal antibody; DAF, decay-accelerating factor; t-PA, tissue plasminogen activator; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

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Expression plasmids were retransfected into 293 cells by the calcium phosphate method. The cells were cultured in serum-free F-12/DMEM for 72 hr. The supernatants (containing the secreted t-PA) were assayed by t-PA ELISA for protein determination.

Biological Assays. The t-PA clot lysis assay was performed as described (14) with concentrated supernatants containing the secreted t-PA. Fluorescein recombination of recombinant human t-PA followed procedures already described (15), using a 2-hr conjugation reaction at 20°C. For the fluorescein isothiocyanate (FITC)-t-PA binding studies, HepG2 cells were plated 24 hr before the start of the experiment. The cells were detached by using 0.04% EDTA, washed three times with phosphate-buffered saline containing 0.5% bovine serum albumin, and stained with FITC-t-PA with or without 100-fold molar excess of unlabeled t-PA for 2 hr on ice. After wards, the cells were washed two times and 1 μl of propidium iodide per ml was added for viable cell gating. For testing the ability of the t-PA mutants to block FITC-t-PA binding, HepG2 cells were incubated with 30 nM mutant t-PA for 2 hr on ice before staining with FITC-t-PA.

FACS. Mouse mAbs were either biotinylated or directly coupled with the fluorescein (FITC) or phycoerythrin (PE) as described (15). Streptavidin–FITC or streptavidin–PE was used to detect binding of biotinylated mAbs. t-PA mAbs were obtained from Genentech (Hybridoma Department). mAb387 is specific for the K1 domain of t-PA, while mAb372 is specific for the protease domain. These two mAbs were isolated and characterized by using either K1 or protease domain deletion mutants of t-PA (B. Fendly, personal communication). The samples were analyzed on a Coulter Elite or 753 flow sorter using 488 nm argon ion laser excitation with emission detected using a 525 ± 25 nm band pass filter for FITC and 575 ± 25 nm band pass filter for PE after electronically removing the propidium iodide staining cells (680 nm long pass filter) as described (11).

RESULTS

Mutagenesis and Library Screening. Previous data had shown that preincubation of t-PA with mAb387 could partially block t-PA binding to rat hepatocytes (B. Keyt and S. Chen, personal communication) and human HepG2 cells (G.C.R., unpublished data) and decrease in vivo clearance of t-PA in rats (B. Fendly, personal communication). This suggested that the mAb387 epitope interacted with the clearance receptor contact domains and that screening for mutants within the mAb387 epitope could lead to isolation of t-PA mutants with longer in vivo half-life and full clot lysis activity.

To screen for K1 domain mutants of t-PA that would no longer bind to mAb387, the expression plasmid pRK-t-PA-DAF was constructed. This vector codes for the production of a fusion protein of t-PA coupled to the last 37 amino acids of DAF. The chimeric protein is targeted and displayed on the cell surface by a glycoprophospholipid membrane anchor. The general scheme for mutagenizing a defined region in the plasmid pRK-t-PA-DAF and for screening t-PA mutants is shown in Fig. 1. A 330-base-pair cassette encoding amino acids 88–188 of the K1 region and 10 amino acids of the K2 domain (5) was randomly mutagenized by PCR conditions of reduced Taq polymerase fidelity (6). The pool of amplified DNA fragments was religated into the vector pRK-t-PA-DAF.

Fig. 1. Scheme for selection of t-PA mutants from randomly mutagenized cDNA libraries using FACS. See text for details.

Fig. 2. Bivariate histograms for isolation of mAb387 epitope loss mutants. TSA201 cells were transfected and 48 hr later stained with mAb387 (PE) and mAb372 (FITC). Sort 1, first transfection of the mutagenized K1 library. Cells were sorted that fell within the top left quadrant, along the diagonal of expressing cells. Episomal DNA was isolated and retransfected (Sort 2). After the second sort, individual clones were analyzed by the two-color immunofluorescence assay. Shown are a mock-transfected control and histograms for mutants k2, k3, k7, k10 (all epitope loss mutants), and K1–6, which displayed a wild-type binding pattern.
Table 1. A collection of 15 K1 mutants that lost the epitope for mAb387

<table>
<thead>
<tr>
<th>Clone no.</th>
<th>Substitution(s) found</th>
<th>HepG2 binding</th>
<th>Clot lysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>k1</td>
<td>Y93C, T103A, N184S, G198D</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>k2</td>
<td>D150V, C168R, Y188C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>k3</td>
<td>N115S, G139R</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>k5</td>
<td>C168S</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>k7</td>
<td>k124E, G137S, D148G, S165T</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>k8</td>
<td>N146I, G198D</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>k9</td>
<td>S127C, Y143H, C173R</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>k10</td>
<td>V157D</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>k11</td>
<td>R130Q, K159R</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>k17</td>
<td>G137D, W154R</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>k18</td>
<td>Q123R, C155R, S169T, N177T, T195A</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>k19</td>
<td>N142S</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>k20</td>
<td>Y156D</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>k22</td>
<td>N146D, L194P</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The mutants are described by a wild-type residue/mutant residue convention; for instance, N142S means that asparagine at position 142 of t-PA has been substituted with serine. ND, not determined.

to generate a library of \(\approx 10^5\) independent clones. The TSA201 cells were transfected and 48 hr later stained with mAb387, directed to an epitope within the mutagenized K1 domain, and mAb372, directed to the nonmutagenized pro tease domain of t-PA. Cells were sorted for the absence of binding to mAb387 and for positive staining for mAb372.

Typical bivariate FACS distributions obtained from transfected TSA201 cells are shown in Fig. 2. From the histogram of the unsorted cells (sort 1), it is clear that most of the mutations did not disrupt the mAb387 epitope, as the expression profile of t-PA on the cell surface is similar to that with nonmutagenized pRK-t-PA-DAF (data not shown). Plasmid DNA was recovered from cells derived from the top left quadrant, amplified, and retransfected. A clearly defined shoulder of mAb387 epitope loss mutants was observed in the second sort. Plasmid DNA was recovered from cells sorted from this region and subsequent transfections were made using DNA derived from individual clones. Over 60% of the isolated clones were found to be epitope loss mutants after two rounds of sorting.

Sequence and Epitope Analysis. DNA sequence comparison of the various K1 epitope loss mutants (Table 1) showed that four clones possessed single point mutations (clones k5, k10, k19, and k20), five clones were generated with two amino acid substitutions (k3, k8, k11, k17, and k22), two clones with three substitutions, (k2 and k9), two with four (k1 and k7), and one each with five (k18) and 10 (k12) point mutations. None of the mutants sequenced contained deletion or insertion mutations. The distribution of the point mutations appears not to be localized to any particular region of the K1 domain based on the predicted three-dimensional structure of K1 of t-PA (B. de Vos, personal communication; ref. 16; Fig. 3). However, as cysteine conversions are expected to disrupt the disulfide bridges, the loss of the mAb387 epitope in clones k1, k2, k5, k9, k12, and k18 may be due to changes in overall folding of K1. As clones k10 (Val-157 to Asp), k19 (Asn-142 to Ser), and k20 (Tyr-156 to Asp) contain single amino acid changes, the mutations found in these clones must be involved in loss of binding of mAb387. Asn-142 is a residue found on the surface of the K1 structure and therefore is likely to be involved directly in mAb387 binding. Tyr-156 and Val-157 are expected to be found in internal positions of K1 near the vicinity of Asn-142; hence, the loss of mAb387 binding in clones k10 and k20 is likely to be due to some local denaturation of the epitope as opposed to specific interaction with mAb387. The three-dimensional structure also predicts that Trp-154 and Asn-146 are located in internal positions of K1 that are distant from Asn-142; clones k17, k22, and k8 with nonconservative changes in these residues are expected to have grossly disrupted K1 structures. The residues Lys-159 in k1 and Asn-115 in k3 are likely to be involved in mAb387 binding, as they are found in external positions in close proximity to Asn-142. None of the four mutated residues found in k7 is in the vicinity of Asn-142; however, Gly-137 and Asp-148 are conserved residues among the various kringle structures (16). Having mutations in these two residues may alter the conformation of K1.

Functional Activity of the Mutants. The individual mutants were subcloned into an expression vector allowing for secretion of the mutant t-PAs for functional studies. The mutants were characterized with respect to their ability to lyse fibrin clots and to bind specific t-PA receptors.

Native t-PA dissolves fibrin-containing clots by converting the inactive zymogen, plasminogen, into the serine protease plasmin via proteolytic cleavage. To assess the effects of the mutations on t-PA activity, the mutants were assayed in a

![Fig. 3. Stereoview of the three-dimensional mapping of the mAb387 epitope on t-PA K1. The α-carbon trace is from the x-ray crystal structure of t-PA K2 (B. De Vos, personal communication). Side chain conformations of K1 are based as closely as possible on those from the K2 crystal structure. Side chains of Asn-115, Asn-142, and Lys-159 are highlighted.](image-url)
fibrin-containing clot assay. The data in Fig. 4 show that most of the K1 mutants are capable of activation of the clot lysis cascade in fibrin matrix with equal or greater than wild-type activity. Only two of the mutants, k12 and k22, possessed <30% of the activity of native t-PA.

The liver appears to be the major organ responsible for removal and catabolism of t-PA (17–19), although the specific cell types responsible for its clearance are as yet unknown. The human hepatoma cell line HepG2 possesses specific high-affinity receptors for t-PA that are capable of internalization and degradation of t-PA (20). Although t-PA can also bind to PAI-1, PAI-1 is located only in the substratum of HepG2 cells and is not detected on the surface of cells in suspension (20). The binding isotherms of total and specific FITC–t-PA binding are shown in Fig. 4. Approximately 33% of the binding was due to nonspecific interactions and the approximate Kd, estimated from the half-maximum value of specific binding, was 7 nM. Both of these values are in excellent agreement with data from Owensby et al. (20) in HepG2 cells using 125I-labeled t-PA. Fig. 4 shows that preincubation of the HepG2 cells with 30 nM wild-type t-PA reduces subsequent FITC–t-PA binding by ~80%. Supernatants from mock-transfected TSA201 cells did not block FITC–t-PA binding. Mutants k3, k19, and k11 with changes in mAb387 binding have reduced abilities to block FITC–t-PA binding relative to wild-type t-PA, suggesting that the mAb387 binding site and the t-PA receptor binding site share common determinants in the K1 domain. The remaining mutants with either cysteine substitutions or mutations at an internal residue might be expected to have reduced binding to the t-PA receptor. The exceptions here are mutants k2 and k7 with either cysteine or conserved residue substitutions, suggesting that t-PA receptor binding may be more tolerant of certain mutations when compared with mAb387 binding.

**DISCUSSION**

We have developed a general method for screening mutants of secreted proteins from randomly mutagenized expression libraries in mammalian cells. This technique was used to map a distinct nonlinear region of t-PA important in recognition of mAb387. In the course of this work, we also isolated t-PA mutants possessing specific altered functional properties. Since t-PA is normally secreted, the expression vector used for screening encoded a t-PA–DAF fusion protein. This construct displayed the library on the surface of transfected mammalian cells. Although not used here, phospholipase C cleavage of the fusion protein at the DAF phosphatidylinositol anchor (7) conveniently releases bioactive t-PA for immediate protein function studies. Expression in mammalian cells is of particular advantage when used to study proteins that require mammalian cell expression for correct conformation—e.g., for proper disulfide bond formation. t-PA, for instance, is not functionally active when expressed in phage or bacteria. Mutagenesis and screening strategies using expression in these foreign hosts therefore may not be particularly relevant for isolation of mutants possessing unusual biological properties.

Expression of the DAF fusion libraries can be screened by any number of approaches. We used FACS to isolate mutants lacking specific antibody binding determinants. The ability to simultaneously measure and sort with two different mAbs by FACS was used to exclude from analysis and sorting cells that did not express surface t-PA and cells that expressed globally misfolded proteins. This approach of sequence mapping can be used to rapidly define regions of structural importance. The mAb387 epitope was mapped using the mutants to a discontinuous region that involves residues Asn-115, Asn-142, and Lys-159 of t-PA.

In general, substitutions in the K1 domain of t-PA appeared to have little effect on fibrin clot lysis. The clot lysis assay measured both the plasminogen activation and fibrin binding activities of t-PA. Not surprisingly, mutant k12 possessing 10 substitutions (including two cysteine substitutions) was only 20% as active as wild type. Mutant k22 also possessed decreased activity from wild-type t-PA. This clone had an Asn-146 to Asp mutation in the K1 domain and a Lys-194 to Pro mutation in the K2 domain. As Asn-146 is expected to be located in an internal position in K1, it is conceivable that placement of a charged residue there would grossly disrupt the t-PA structure.

In contrast to the overall lack of effect of K1 substitutions on clot lysis activity, substitutions in the K1 domain appeared to have marked effects on the ability of the mutants to bind to the t-PA receptor expressed in HepG2 cells. All of the mutants with changes in the mAb387 epitope that involves amino acids Asn-142, Asn-115, and Lys-159 also had reduced binding to the t-PA receptor as compared to wild-type t-PA. It will be of interest to determine whether t-PA with mutations in these three residues would possess a longer half-life in vivo. Using domain deletion mutants, previous investiga-
tors have shown that in vivo clearance is decreased by deletion of the finger and growth factor domains (21, 22) or removal of glycosylation sites (21). Our data on K1 mutants as well as the decreased rates of clearance in rats after mAb387 binding to t-PA (B. Keyt and S. Chen, personal communication) suggest that the mAb387 epitope in the K1 domain may also be involved in clearance receptor binding.

In summary, the PCR/FACS random mutagenesis methodology using a vector containing a membrane anchor sequence permits rapid sorting of secreted proteins directly by expression in mammalian cells. The method is useful for mapping nonlinear structural domains involved in antibody binding as well as functional properties that are associated with antibody binding to the molecule. Several minimally substituted K1 domain t-PA mutants having decreased ability to bind to the putative hepatic t-PA clearance receptor in vitro were isolated. This method should be useful for structure-function analysis of protein interactions and for the design of mutant proteins with unusual properties.

We thank Dr. Bart de Vos for providing us with the three-dimensional structure of K2 of t-PA and Dr. Leanard Presta for providing us with the K1 three-dimensional model of t-PA and for helping us interpret the location of the various amino acids in the three-dimensional structure. We are grateful to Dr. Bruce Keyt and Sharon Chen for sharing their data on mAb387 and to Brian Fendly for supplying us with the antibodies used in this study.