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Technical note

A rapid screening method for cell lines producing singly-tagged recombinant proteins using the "TARGET tag" system

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ABSTRACT

Recombinant production of extracellular glycoproteins in stable mammalian cell lines is an ideal technique for obtaining a large quantity of high-quality proteins. In most cases, however, current methodologies do not allow for sufficiently rapid cell line development and protein purification. Here, we describe a 21-residue peptide tag (designated as TARGET tag) and its use for rapid stable cell line development and purification. The ability of the anti-tag antibody P20.1 to withstand repetitive regeneration cycles has enabled the development of a sensitive surface plasmon resonance-based screening format that requires only 20 μl of cell culture supernatants. Direct and semi-quantitative screening at the 96-well culture scale eliminated the need for a second screening, re-cloning, or sorting, thereby minimizing culture pre-production time. Using this system, "high producer" cell lines were established in less than a month, and milligram quantities of target proteins could be purified with a standardized protocol.

1. Introduction

The demand for functional and structural analyses of proteins is only increasing as we enter the post-genomic era. Accordingly, there is a high demand for the development of simpler and faster methods to achieve production/purification of high-quality recombinant proteins. Among the many different expression systems that are currently in use, depending on the nature of the target, mammalian cell expression is particularly suitable (and sometimes even obligatory) for the production of extracellular proteins of mammalian origin, due to the requirement of unique posttranslational modifications and specific chaperones. However, the laborious and time-consuming nature of this procedure, as well as the high cost associated with it, preclude this method from gaining much popularity in research laboratories.

Several groups have reported optimized protocols for time- and cost-efficient protein production using a transient transfection of mammalian cells [1–5]. Of particular note, the slow growth rate intrinsic to mammalian cells was circumvented by utilizing large-scale suspension culture system in some studies. However, such a transient system lacks sustainability and cannot provide a constant source of recombinant proteins. Production of stable transfectants, on the other hand, is an excellent choice when a relatively large quantity of target protein is required over a period of time, rather than in a

Abbreviations: GFP, green fluorescent protein; mAb, monoclonal antibody; PAR-4, protease activated receptor-4; SPR, surface plasmon resonance; TBS, Tris-buffered saline; HEK, human embryonic kidney.

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"one-shot" experiment. There exists here, as well, a significant drawback since one has to select and isolate a cell clone that highly expresses the target protein. Mancia et al. [6] have developed an excellent system to isolate stable cell lines producing membrane proteins or secreted proteins using green fluorescent protein (GFP[1]) coexpression. The time required for obtaining the final cell line, however, is still rather long (~2 months) since this procedure involves cell sorting or cloning.

On the purification side, mammalian expression systems also possess some intrinsic shortcomings. Generally, the level of recombinant proteins secreted from mammalian cells is, at best, in the range of mg per L, while the abundance of the target protein is less than 0.1% due to the presence of serum in the culture media. As a consequence, initial affinity chromatography suffers from the incomplete capture of the target protein, as well as severe contamination from these weakly binding serum-derived proteins. Recently, we have developed a peptide-based affinity tagging system that is highly compatible with protein purification from cell culture supernatants [7]. This system utilizes a monoclonal antibody (mAb) P20.1 against a 6-residue peptide from the N-terminal portion of human protease-activated receptor-4 (PAR-4) [8]. The low affinity towards the peptide inherent to this mAb (K_D=1.5 μM) can be overcome by the use of a tag peptide carrying x3 concatenated epitopes (i.e., 18 residues). This system has been successfully applied to the one-step purification of human F-spondin from cell culture supernatant [9]. The biggest advantage of this system over other existing anti-peptide antibody purification systems is the very mild elution conditions required for antibody-captured antigen using 40% propylene glycol. This water-miscible organic solvent does not have a denaturing effect on proteins and thus the purified proteins retain their structural and functional integrities [7]. A group of mAbs that show similar polyol-sensitive properties have been reported and the cognate peptide tags were commercialized under the name Softag [10,11].

In the present work, we optimized both the sequence and the length of tag peptide, yielding a 21-residue peptide with a K_D of 10 nM. The resultant tag (designated as TARGET tag for tandemly-arranged recognition motif combined with gentle elution technology) was evaluated for the use in protein purification and screening. The gentle nature of the elution condition ensured the successful repetitive use of the antibody column for purifying tagged proteins. Furthermore, the sensitive detection of tagged proteins in the culture supernatants and the mild regeneration conditions enabled the establishment of a surface plasmon resonance (SPR)-based high-throughput screening method that can significantly shorten the time required for obtaining a high producer cell clone.

2. Materials and methods

2.1. Construction of phage display libraries

A combinatorial library of the 7 amino acid peptide fused at the N-terminal of the pIII coat protein of the M13 filamentous phage was prepared using the pComb3 system by the method described previously [12]. To introduce random mutations at residues 1, 3, 4, 6, and 7, while retaining Tyr2 and Gln5, we synthesized the 5′-primer with randomized codons, 5′-CATGCCATGGCCNNKTACNNKNNKCAANNKNNKGGAGGCTCGAGCGTAAG-3′ (the NcoI site is underlined) and the 3′-primer, 5′-CTTACGCTCGAGCCTCC-3′ (the XhoI site is underlined). PCR was carried out with 30 cycles of denaturing for 60 s at 96 °C, annealing for 30 s at 55 °C, and extending at 72 °C for 30 s. The PCR product was cloned into the NcoI–XhoI site of pComb3, followed by the transformation into *E. coli* XL1-blue by electroporation. The phage-displayed library was prepared as previously described [12].

2.2. Phage selection

The phage library was selected against P20.1 antibody coated onto a 96-well microtiter plate (NUNC Maxisorp). Wells were coated with 5 μg/ml P20.1 IgG at room temperature for 2 h and blocked with phosphate-buffered saline containing 5% bovine serum albumin. Fifty microliter of freshly prepared phage (2×10^{10} °CFU) was added to each well, incubated for 10 min at room temperature, and washed extensively with wash buffer (50 mM Tris, 400 mM NaCl, 0.05% (v/v) Tween 20, pH 7.5) 20 times over a 3 h period. Rapidly growing XL1-blue *E. coli* cells at the logarithmic phase were then directly added to the well (100 μl/well), incubated for 30 min at 37 °C to allow for infection, and further grown in 14-ml culture tubes. For the next round of panning, the phage was rescued by adding the helper phage VCSM13. In each round, *E. coli* infected with the P20.1-bound phage was spread onto LB-agar plates and individual clones obtained after overnight incubation at 37 °C were subjected to pComb3 plasmid preparation and sequencing using M13 primer.

2.3. Surface plasmon resonance analysis of fusion proteins

Fusion proteins carrying concatenated P20.1 epitope peptide were prepared using a design similar to that described previously [8]. Briefly, a DNA fragment coding for human fibronectin (comprising the 9th to 10th type III repeats preceded by the tag sequence) was cloned into the NdeI–BamHI site of pET16b vector (Novagen). As a result, each construct carried the N-terminal Hisx10 tag (MGHHHHHHHHHHSSGHIEGRHM), followed by either the GYPGQVGYPGQVGYPGQV (GYPGQVx3) or YPGQYPGQYPGQYPGQYPGQV (YPGQx5+V) peptide. The fusion proteins were expressed in *E. coli* BL21(DE3) and purified by Ni-NTA chromatography. For SPR analysis, P20.1 IgG was immobilized on the CM5 sensorchip using amino coupling chemistry according to the method provided by the manufacturer. Briefly, P20.1 IgG dissolved in 50 mM sodium acetate buffer (pH 5.0) at 20 μg/ml was injected over the CM5 chip activated for 7 min at a flow rate of 10 μl/min. Typically, infusion of 30 μl of this antibody solution results in ~500 RU immobilization. Fusion proteins (1–2 mg/ml in 20 mM Tris, 150 mM NaCl, pH 7.5 (Tris-buffered saline; TBS)) were diluted more than 500-fold with the running buffer (20 mM Hepes, 150 mM NaCl, 0.01% (v/v) Tween 20, pH 7.2) and injected at a flow rate of 20 μl/min. All SPR experiments were performed using a BIACORE 2000 instrument (GE Healthcare).

Table 1 – Amino acid sequence of peptide recognized by P20.1 mAb.

		Sequence[a]							
	ID	1	2	3	4	5	6	7	Freq.[b]
Series 1	1	D	Y	P	G	Q	V	C	1/14
	2	I	Y	P	G	Q	W	G	1/14
	3	Q	Y	S	G	Q	W	P	1/14
	4	L	Y	V	G	Q	V	G	1/14
	5	S	Y	S	G	Q	V	H	1/14
	6	Y	Y	S	G	Q	V	Q	1/14
	7	S	Y	P	G	Q	M	N	1/14
Round 1	8	R	Y	S	G	Q	V	G	1/14
	9	V	Y	P	G	Q	L	N	1/14
	10	R	Y	P	G	Q	T	G	1/14
	11	W	Y	P	G	Q	L	E	1/14
	12	G	Y	C	V	Q	L	Q	1/14
	13	T	Y	P	G	Q	L	E	1/14
	14	S	Y	Q	G	Q	W	T	1/14
	11	W	Y	P	G	Q	L	E	9/14
	15	M	Y	T	G	Q	T	V	1/14
Round 2	16	K	Y	G	G	Q	V	P	1/14
	17	V	Y	P	G	Q	Y	R	1/14
	18	A	Y	Q	G	Q	V	P	1/14
	19	G	Y	P	G	Q	W	S	1/14
Series 2	11	W	Y	P	G	Q	L	E	3/28
	20	L	Y	P	G	Q	A	V	2/28
	21	R	Y	V	G	Q	I	P	1/28
	22	T	Y	P	G	Q	V	R	1/28
	23	L	Y	P	G	Q	V	V	1/28
	24	S	Y	P	G	Q	V	G	1/28
	25	V	Y	D	G	Q	I	S	1/28
	26	R	Y	P	G	Q	V	A	1/28
	27	R	Y	A	G	Q	V	P	1/28
	28	T	Y	P	G	Q	E	E	1/28
	29	L	Y	A	G	Q	V	P	1/28
	30	R	Y	P	G	Q	V	D	1/28
Round 1	31	L	Y	E	A	Q	Q	M	1/28
	32	W	Y	T	G	Q	A	P	1/28
	33	S	Y	A	G	Q	I	G	1/28
	34	M	Y	A	G	Q	Y	V	1/28
	35	R	Y	P	G	Q	L	L	1/28
	36	G	Y	S	G	Q	V	G	1/28
	37	S	Y	P	G	Q	V	M	1/28
	38	M	Y	S	G	Q	V	P	1/28
	39	F	Y	G	E	Q	S	M	1/28
	40	Q	Y	P	G	Q	V	I	1/28
	41	H	Y	P	G	Q	A	W	1/28
	42	R	Y	P	G	Q	M	S	1/28
	43	D	Y	D	G	Q	F	P	1/28
Round 2	11	W	Y	P	G	Q	L	E	7/7

[a]Clones were randomly picked from the pool of phage obtained after each panning round and sequenced. Independent clones having a particular DNA sequence are assigned with a unique ID number. Fixed amino acid positions are shown in gray background.
[b]Frequency of the occurrence of each sequence during the same round.

2.4. *P20.1-Sepharose affinity purification of GFP_{UV}*

DNA fragment coding for GFP_{UV} with YPGQx5+V peptide (the TARGET tag) attached at the N-terminus was prepared by extension PCR using $pGFP_{UV}$(Clontech) as the template, and cloned into pET16b vector. TARGET-GFP_{UV} was produced in transformed *E. coli* BL21(DE3) culture for 6 h in the presence of 1 mM IPTG, and the bacterial pellet from a 700 ml culture was suspended in 30 ml of TBS in the presence of a protease inhibitor cocktail. Bacteria were lysed by sonication and the soluble fraction was obtained by centrifugation at 15000×*g* for 30 min. The lysates were passed through a 0.45 μm filter to remove any trace amounts of insoluble materials, and aliquots were stored at −80 °C until later use. Small-scale purification of GFP_{UV} was carried out as follows. A mini column containing 0.5 ml of P20.1-immobilized Sepharose prepared as previously described [7] was equilibrated in TBS. Bacterial lysate (0.25 ml) was then applied onto the column, followed by a washing step (2 ml of TBS) and an elution step (2.5 ml of 40% (v/v) propylene glycol/TBS). Monitoring the fractions using a hand-held UV lamp (365 nm) confirmed both the complete capture of the GFP_{UV} to the column and the full recovery of the bound GFP_{UV} in the first 2 ml elution fraction. The GFP_{UV} contained in the pooled eluate fraction from each purification cycle was quantified with a fluorescence measurement at excitation and emission wavelengths of 390 and 510 nm, respectively, using a Hitachi F-7500 fluorescence spectrophotometer. All chromatographic procedures were performed at 4 °C.

2.5. *Stable transfection of HEK293T cells and high-throughput screening*

Expression of mouse semaphorin 6A (sema6A) ectodomain fragment was conducted as follows. A DNA fragment coding for residues 1 to 552 of mouse sema6A followed by a TEV protease recognition sequence and a TARGET tag sequence was prepared by extension PCR, and cloned into pcDNA3.1 vector (Invitrogen). The resulting plasmid was mixed with 1/10 amount of pEF1-puro plasmid [13] and transfected into rapidly growing human embryonic kidney (HEK) 293T cells plated in a 6-well plate, using Fugene 6 transfection reagent (Roche) according to the manufacturer's protocol. Twenty-four hours later, cells were detached using 0.05% (w/v) trypsin/0.5 mM EDTA (Gibco) and suspended in a complete medium (DMEM +10% FCS) containing 10 μg/ml puromycin (Sigma) and plated into 96-well culture plates at various dilutions (typically 3000 cells/well). Untransfected cells died out within 3 to 5 days, and surviving cell colonies appeared at around day 8. On day 13, ~50 μl of culture supernatant from the wells developing a single colony were collected, and were subjected to the SPR-based binding assay. For the binding assay, CM5 sensorchip immobilized with P20.1 IgG at ~3000 RU was used. Binding was evaluated by injecting 20 μl of culture supernatant at a flow rate of 20 μl/min, followed by a 20-μl injection of 40% (v/v) propylene glycol in the running buffer. Cell clones identified as "high producers" were subjected to stepwise culture expansions, and finally cultured in 10 large culture flasks (T225, Corning) to obtain 300 ml of conditioned media on day 30. Cell lines producing mouse plexin A2 ectodomain fragments (residues 1 to 561) and mouse NPP2 full-length proteins (residues 1 to 863) were established using the same procedure described above, except that HEK293S GnT1$^-$ cells [14] were used for the transfection and selected against 1 mg/ml G418 (Gibco). In addition, these cells were finally cultured in a

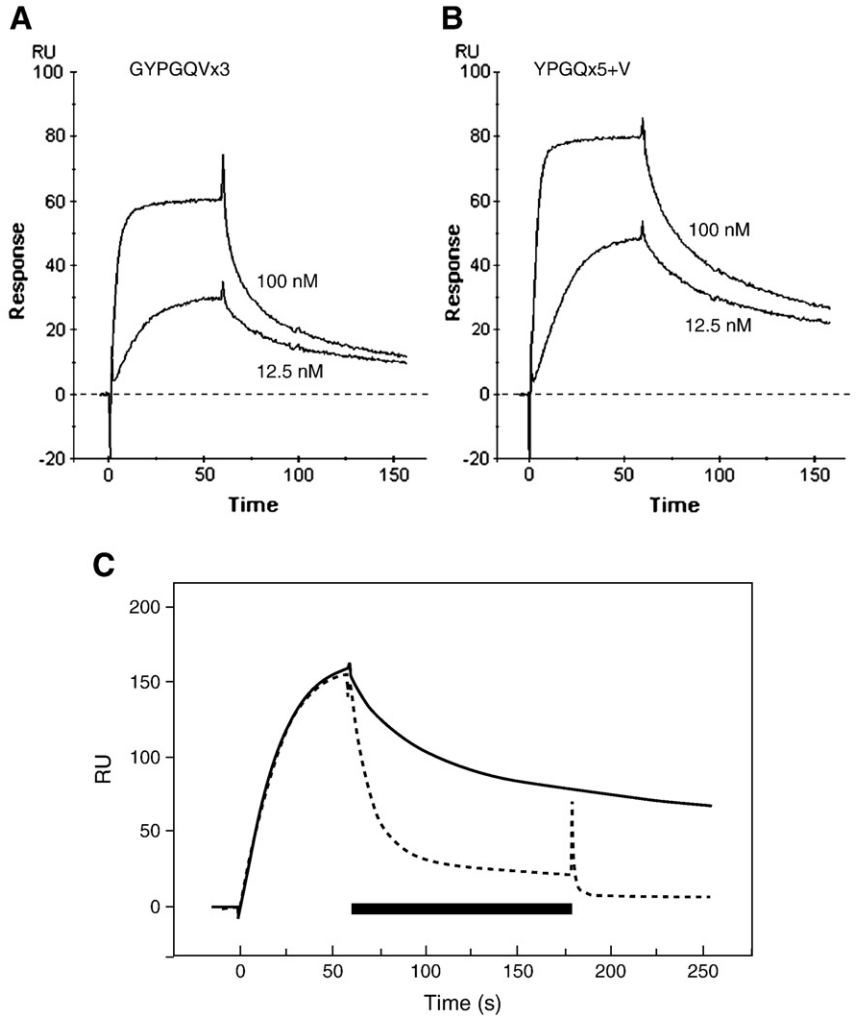

Fig. 1 – Surface plasmon resonance analysis of the revised tag peptide. Sensorchip was immobilized with P20.1 IgG (~500 RU) and Fn9-10 proteins N-terminally tagged with the 18-residue, GYPGQVx3 peptide (A) or the 21-reside, YPGQx5+V peptide (B) were infused at the indicated concentration. Steady-state binding analysis using Req values obtained at five different concentrations derived the apparent K_D of 19 nM and 10 nM for the 18- and 21-residue peptides, respectively. (C) Fusion protein with the YPGQx5+V peptide tag was made to flow over the P20.1-sensorchip at 25 nM for 60 s, followed by an infusion of the 100 μg/ml PRGYPGQV peptide (dashed line) or running buffer only (solid line) during the next 120 s. Note that although the peptide quickly reversed the binding of the tagged protein, it produced a sustained binding signal of ~20 RU due to its own binding to the antibody.

BelloCell™ bioreactor (CESCO Bioengineering) [15] connected to a media circulating system with a 2.5 l reservoir.

2.6. *Purification of recombinant proteins*

Culture supernatants of cells stably expressing recombinant proteins were clarified by centrifugation and the pH was adjusted to 7.5 with 1 M Tris. P20.1-Sepharose was then added to the solution (1 ml beads/100 ml culture supernatant), and the mixture was incubated for 2 h at 4 °C with continuous rotation of the container. The beads were then transferred to an Econo-Column (1.5×15 cm, Bio-rad), washed with 5 column volumes of TBS, and eluted with 5 column volumes of TBS containing 0.2 mg/ml PRGYPGQV peptide over a period of 30 min. The elution fractions were combined, dialyzed against 20 mM Hepes, 150 mM NaCl, pH 7.5, and concentrated using ultrafiltration (Corning SpinX UF, MWCO 30,000). Typically, the entire purification process was completed within 2 days.

3. Results and discussion

Previously, we determined that the recognition motif for mAb P20.1 is fully contained in a 6-residue portion (GYPGQV, referred to as P4 sequence) of human PAR-4 N-terminal peptide [8]. Furthermore, Ala scanning mutagenesis has revealed that the Ala substitution at three positions (i.e., Gly1, Pro3, and Val6) was tolerated. In order to obtain an optimized and minimum sequence motif recognized by P20.1, we constructed a peptide library with a partially randomized P4 sequence and screened it for P20.1 binding using a phage display strategy. The library was

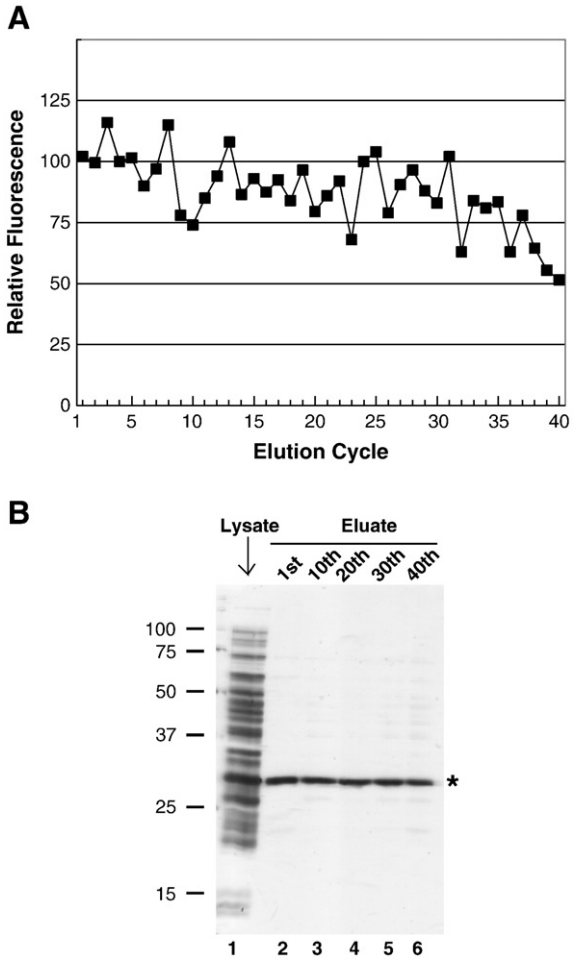

Fig. 2 – Purification of TARGET-tagged GFP_{UV} using P20.1-Sepharose. (A) Capacity change during forty consecutive runs. A small column containing 0.5 ml P20.1-Sepharose was used to purify tagged GFP_{UV} from a bacterial lysate and the GFP_{UV} contained in the elution fraction of each run was quantified fluorometrically. (B) Quality of purified GFP_{UV}. The eluted materials from the 1st (lane 2), 10th (lane 3), 20th (lane 4), 30th (lane 5), and 40th (lane 6) runs were subjected to SDS-PAGE followed by silver staining. Note that only the 28-kDa band corresponding to GFP_{UV} (asterisk) is bound to and eluted from P20.1-Sepharose, among the many proteins present in the bacterial lysate (lane 1).

constructed using a pComb3 system and prepared by PCR, in which the codons for G1, P3, G4, V6, and an additional 7th residue were randomized (Table 1). The codon for G4, which has been shown to be important for recognition, was randomized to validate the selection procedure. By transforming the pComb3-P4 variants we obtained a library of 6.3×10^6 clones, which was greater than the possible combinatorial number of five amino acids, 3.2×10^6. We performed two independent selection series, each involving two rounds of panning with P20.1 IgG. After each round, randomly picked clones were sequenced. Among the 63 clones that were sequenced, 60 clones (95%) had Gly at position 4, confirming the specificity of the selection procedure employed. To our surprise, one specific peptide sequence (WYPGQLE) became highly enriched after only two rounds of panning in two independent screening, suggesting that it represents a high affinity motif. The binding kinetics of the fusion protein carrying this motif, however, did not show any sign of improvement from the original sequence in an SPR assay (data not shown). Furthermore, all clones coding this particular peptide sequence had identical nucleotide sequences. We speculate that the enrichment of this sequence was not due to its high affinity trait, but probably stems from the relative abundance of the clone in the original library. Due to the library saturation caused by this clone after the second panning, we did not perform additional selection cycles. Instead, we focused on analyzing the frequency of particular amino acid occurrence at specific positions. Among the randomized positions, a wide variety of amino acids were found at the 1st and the 7th positions, suggesting that these two positions do not in any way contribute to the recognition by P20.1. In contrast, the 3rd and the 6th positions showed a clear tendency for selecting small and hydrophobic amino acids, respectively (Table 1). Pro was particularly favored at position 3, consistent with the curved backbone configuration of the antibody-bound peptide revealed by the crystal structure [7]. At position 6, 39 out of 43 independent peptide sequences isolated had hydrophobic residues. This was rather unexpected since the antibody surface near the Val6 of the bound peptide was not particularly hydrophobic in nature [7].

The phage display screening described above established that the ideal peptide motif required for P20.1 binding is defined as YPGQϕ, where ϕ represents any hydrophobic amino acids. We utilized this information to re-design our purification tag sequence. The current tag sequence is an 18-residue peptide containing a triplicated P4 sequence (i.e., GYPGQVGYPGQVGYPGQV). As the phage display experiment showed that the first G of the repetitive unit is dispensable, the unit can thus be shortened to YPGQV. Furthermore, the last V can be removed from the repetitive unit because the first residue of the next repeat, Y, would play a similar role. In other words, the general design of the tag sequence can be written as $(YPGQ)_n+V$. We tested this using a 21-residue peptide ($n=5$) attached N-terminal to a model protein Fn9-10 fragment. As shown in Fig. 1A, the newly designed peptide does, indeed, have the expected property; i.e., fusion protein with the revised tag sequence bound to the immobilized P20.1 with an apparent affinity of 10 nM, which is 2 times higher than that of the original 18-residue sequence (19 nM). This increase in affinity was mainly due to the decreased initial off-rate ($0.026\ s^{-1}$ *vs.* $0.049\ s^{-1}$). As the true monovalent affinity of P20.1 towards the P4 sequence is low (1470 nM) [7], the high apparent affinity of the tag peptide containing the multiple motifs is likely to be caused by the frequent binding-rebinding cycles that typically occur on a linear stretch of peptide. This was confirmed by the experiment shown in Fig. 1B, where excess free antigen peptide was added during the dissociation phase of the SPR (thick horizontal bar). The quick reversal of the SPR signal by the peptide corroborates the presence of rebinding, which constitutes the mechanism of affinity-enhancement by motif concatenation. This is also consistent with the observation that the increased number of motifs (5 *vs.* 3) and the closer apposition (4-residues *vs.* 6-residues) contributed favorably to the binding affinity of the redesigned peptide. We have designated the 21-residue peptide 'TARGET

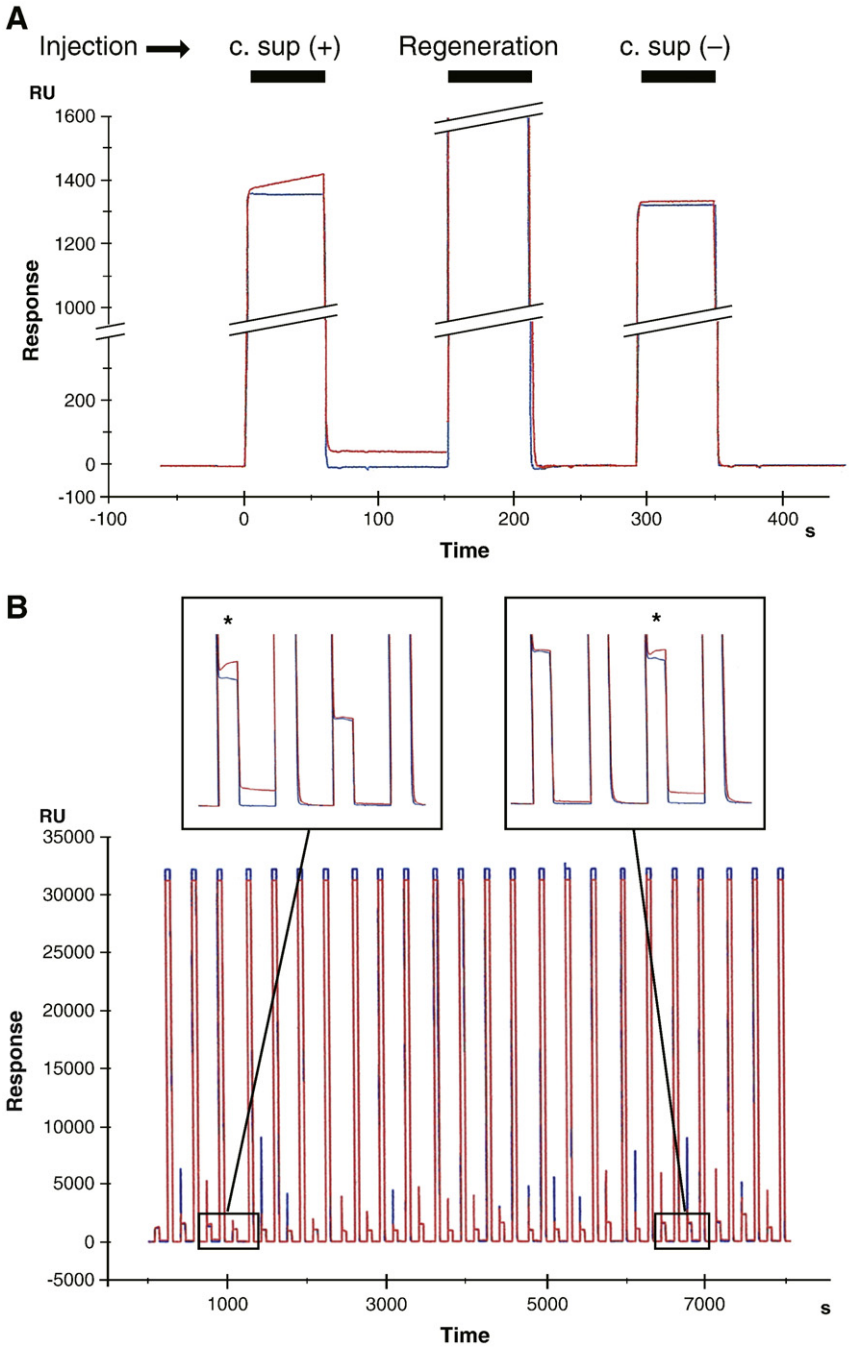

Fig. 3 – SPR-based screening of cell clones expressing TARGET-tagged proteins. (A) Sensorgrams were recorded on surfaces immobilized with P20.1 (red) or control IgG (blue) during the consecutive injections of culture supernatant containing TARGET-tagged protein (c. sup (+)), running buffer containing 40% propylene glycol (Regeneration), and control culture supernatant (c. sup (–)). Note that the signal corresponding to the bound protein on P20.1-coated surface (~ 60 RU) returns to a baseline level after the injection of 40% propylene glycol. (B) Actual sensorgrams from the screening process. Undiluted culture supernatants (20 μl each) from sema6A-expressing stable cell clones grown in 96-well plates were injected, followed by a pulse regeneration (20 μl) by 40% propylene glycol. Portions of sensorgrams near the two highly expressing clones (asterisks) are magnified and shown at the top.

tag' for tandemly-arranged recognition motif combined with gentle elution technology.

We next tested whether the new tag sequence was compatible with the one-step purification system using P20.1-immobilized beads. The unique nature of the P20.1 antibody is the complete reversal of its peptide binding in the presence of >40% water-miscible organic solvents such as propylene glycol [7]. Such mild elution conditions would not only maintain the structural and functional integrity of the eluted proteins, but also prolong the life of the antibody resin. We fused a TARGET tag to the N-terminus of GFP_{UV} and expressed it in *E. coli*. Application of the bacterial lysate to a small (0.5 ml) column of P20.1-Sepharose resulted in complete capture of the fluorescent protein, and the bound TARGET-

Table 2 – Time required for the actual protein production projects.

Protein	Cell	Days after cell transfection [a]					Culture sup (l)	Yield [b] (mg/l)
		SPR screening	Cell expansion	Large-scale culture	Purification	Total		
sema6A (fragment)	293T	13	23(10)	30(7)	32(2)	32	0.3	1.6
PlexinA2 (fragment)	293GnT1-	23	48(12)	62(13)	64(2)	50	5	4.9
NPP2(full-length)	293GnT1-	22	49(10)	66(15)	97(2)	49	5	5.2

[a] Chronological number of days passed following cell transfection when each step was completed. The actual days required for each step are indicated in parentheses, and the sum of these numbers corresponding to the net "operating" days are shown as the total.
[b] The amount of final purified proteins per liter of culture supernatants.

GFP_{UV} was successfully eluted with a buffer containing 40% propylene glycol. Importantly, complete purification was achieved in one step (Fig. 2B, lane 2). In order to evaluate the effect of the propylene glycol treatment on column performance, the same purification cycles were repeated using the same column. The GFP_{UV} recovery in each run was then quantified by measuring the fluorescence intensity of the eluted fraction. Although the GFP_{UV} recovery varied each time, a very high average column capacity (~60% of the initial value) was retained even after 40 binding/elution cycles (Fig. 2A). This converts to less than 1% activity loss per cycle, and clearly shows that repeated exposure to the elution buffer (40% propylene glycol) has a minimal effect on the antibody's binding capacity. In addition, the quality of the eluted material did not diminish as judged by SDS-PAGE analysis (Fig. 2B), indicating that both the specificity and affinity of P20.1 antibody were preserved. This gradual loss of the activity more likely stems from the accumulation of insoluble materials present in the bacterial lysate inside the Sepharose beads, rather than from damage done to the antibody.

The ability to withstand repeated elution (regeneration) cycles is not only ideal for a purification system, but also a desired property for a screening probe. For successful recombinant protein production using a stable mammalian cell line, selecting a cell clone that produces target protein at high levels is critical. Generally, this involves an initial screening and subsequent re-cloning and/or sorting of the cells, taking weeks until the cells are ready for production-scale culture. A simple and sensitive assay for the presence of target proteins in the 96-well culture supernatant would eliminate the need for a second culture expansion and the selected cell clone could be directly transferred to a large-scale culture. Although ELISA-based screening is desirable for this purpose, singly-tagged proteins are not compatible with ELISA detection unless specific antibody against the target protein itself is available. Thus, we utilized the P20.1-TARGET tag system in a cell-line screening method using an SPR-based high-throughput assay format. Proteins were tagged with the TARGET tag and cell culture supernatants were made to flow directly over the P20.1-immobilized sensorchip. As shown in Fig. 3A, culture supernatant of cells secreting TARGET-tagged model protein generated a positive signal on the sensorgram, while the control supernatant showed no sign of binding. Although the serum-containing culture supernatants made a huge spike of ~1300 RU in the sensorgram because of the refractive index change, a comparison with the control surface (blue lines in Fig. 3) allowed us to easily identify positive samples by noting the ascending resonance signal during the sample infusion and the sustained value during the buffer wash. Importantly, the bound tagged proteins can be completely removed by the pulsed infusion of 40% propylene glycol, making the surface ready for the next injection.

We applied this method in the screening of the cell line producing soluble ectodomain fragment of mouse semaphorin 6A (sema6A). HEK293T cells were transfected with plasmid coding for sema6A with the TARGET tag attached at the C-terminal together with selectable marker plasmid (pEF1-puro) and cultured in 96-well plates in the presence of 10 μg/ml puromycin. After 2 weeks, culture supernatants from wells containing a single colony were harvested and immediately subjected to SPR screening with the P20.1 antibody. Fig. 3B shows the raw sensorgram obtained during the 24 consecutive injections of 20 μl culture supernatants, from which positive clones could be easily identified (asterisk). Forty-eight clones were analyzed over two rounds of SPR analyses in one day, and clones with the highest signal were immediately expanded to a larger culture. Continuous culture expansion of the selected cell clone minimized the time required to obtain large quantities of culture supernatants. Finally, we obtained 300 ml of culture supernatant on day 30 following transfection (Table 2). Tagged sema6A was purified from the culture supernatants using affinity chromatography on P20.1-Sepharose (Fig. 4A, left panel), and complete purification was achieved in one step (Fig. 4A, right panel). A total of 0.49 mg purified sema6A fragment was obtained from 300 ml of culture supernatant, and the purified protein was biologically active in a cytokine production assay using rat dendritic cells (data not shown).

The same screening/purification procedure using P20.1 and the TARGET tag was further applied to two more projects aimed at recombinant production of an ectodomain fragment of semaphorin receptor plexinA2, and a secreted enzyme nucleotide pyrophosphatase/phosphodiesterase 2 (NPP2, also known as autotaxin). Both proteins were C-terminally TARGET-tagged and transfected into HEK293S GnT1$^-$ cells. The procedures were essentially identical to that utilized for sema6A, except that the stable transfectants were cultured in a high-density cell culture bioreactor (Bello Cell™) to collect large volumes of the culture supernatants. As shown in Fig. 4B and C, both proteins were successfully purified from the supernatants using P20.1-Sepharose in just one step, and the final yield was ~5 mg/l. Table 2 shows the actual time (days) required for each project. In the case of the "rush" project (e.g., sema6A), it was possible to obtain mg quantities of protein in

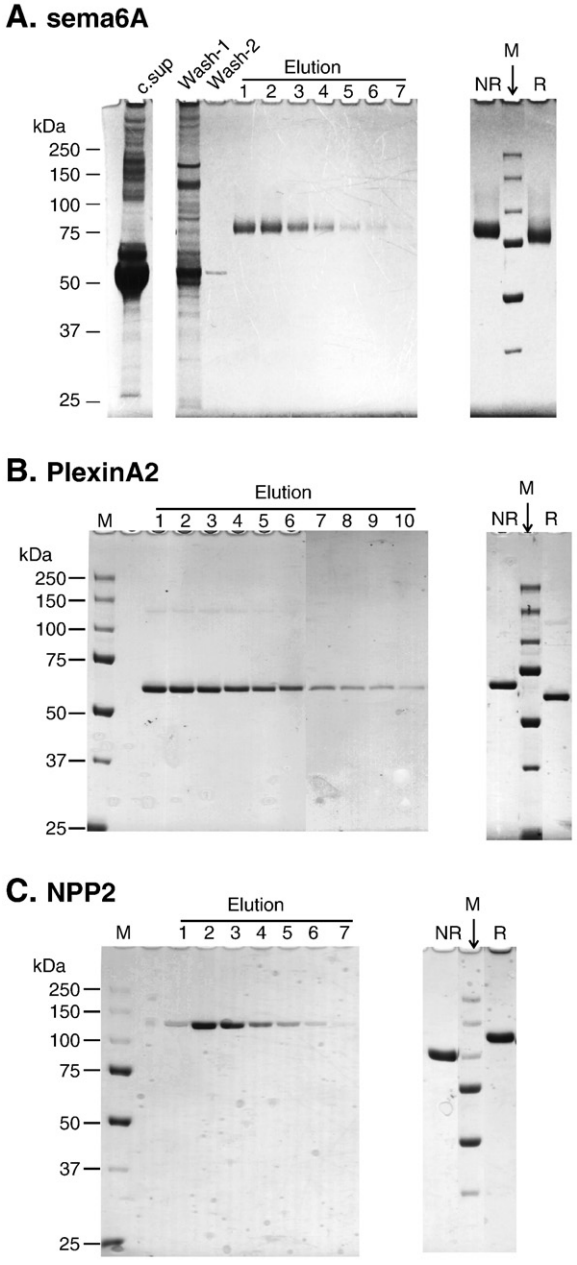

Fig. 4 – One-step purification of recombinant proteins C-terminally tagged with TARGET tag. (A) Purification of sema6A. Ten microliters of the first and the second wash fractions, as well as the seven elution fractions from the P20.1-Sepharose chromatography were subjected to 10% SDS-PAGE under nonreducing condition and stained with Coomassie blue. Five microliters of the starting material, the culture supernatant of the 293T cell clone expressing the sema6A fragment (c. sup), was also analyzed. Elution fractions were dialyzed and concentrated and re-analyzed on the nonreducing (NR) or reducing (R) SDS-PAGE. Note that the eluted material is essentially free of contaminants. (B) and (C) Purification of plexin A2 (B) or NPP2 (C). The purification procedure was essentially identical to that described in (A), and Coomassie blue-stained SDS-PAGE gels of elution fractions (left panels) and purified materials (right panels) are shown. Positions for the molecular weight markers (M) are shown at the left.

about a month by minimizing the idle time between the different experimental steps. In the case of plexin and NPP2, the process took slightly longer due to the slow growth of 293S GnT1$^-$ cells and the inclusion of a high-density cell culture step. Still, more than 10 mg of purified protein could be obtained in ~50 days.

In the present study, we have demonstrated the followings: (1) the recognition sequence of P20.1 antibody can be generalized to YPGQϕ, where ϕ represents any hydrophobic amino acids, and overlapping motif concatenation resulted in a 21-residue peptide (YPGQYPGQYPGQYPGQYPGQV) designated the 'TARGET tag'; (2) mild elution conditions (i.e., 40% propylene glycol) resulted in a surprising degree of antibody column longevity; and (3) P20.1 was implemented in a SPR-based direct screening system for cell culture supernatants and enabled very rapid cell line development for the production of proteins of interest. The biggest advantage of the SPR-based screening is the elimination of cell expansion/re-cloning step. It requires only ~20 μl culture supernatant, allowing the early and direct screening of cells at the 96-well stage. This makes the procedure much like the hybridoma screening in a monoclonal antibody development. Although ELISA-based screening would be preferred because of the ease in the experimental settings, suitable antibody against the target protein is not always available. Furthermore, one has to use sandwich ELISA format for such screening, necessitating the attachment of two independent tags at sterically compatible locations. TARGET tag can be fused with either N- or C-terminus of proteins, along with a protease-cleavage site if tag removal is necessary. Similar SPR-based screening for conventional (e.g., FLAG) tag is theoretically possible but is impractical because one has to use the anti-tag antibody immobilized indirectly (through the capturing secondary anti-mouse antibody) which should be removed and re-loaded each time before the next sample injection. In contrast, mild yet complete regeneration of P20.1-immobilized surface eliminates such cumbersome and potentially expensive procedures, allowing the completion of a screening process in a day. The effect of pulse injected 40% propylene glycol on the P20.1-immobilized sensorchip during the regeneration step appears negligible, since the sensitivity of the surface remained unchanged after more than 200 injections were made (data not shown). The SPR-based method can reliably detect ~0.1 μg/ml tagged antigen in the undiluted, serum-containing culture supernatant (data not shown). This sensitivity is not necessarily high compared to other methods such as ELISA. Considering the level of expression that is required for stable cells for protein production, however, the demand for a higher sensitivity during the screening would be low. Furthermore, the semi-quantitative nature of the SPR-based assay allows one to use it for monitoring the production of recombinant proteins in transient expression, particularly when optimizing the expression constructs and/or the duration of the culture after the transfection.

To date we have applied this screening/purification method to 8 different projects, all of which led to the successful establishment of stable cell lines secreting high levels of target protein in 3–5 weeks. From the cell culture supernatants obtained thus far, recombinant proteins were subsequently purified to homogeneity using P20.1-Sepharose,

without any prior processing such as dialysis or concentration. The purified proteins could be used in functional assays or crystallizations for structure determination. In conclusion, the use of P20.1 antibody in combination with the optimized 21-residue tag peptide offers a very efficient method to develop stable cell lines for the recombinant production of biologically important proteins.

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