

The construction and characterization of a bifunctional EGFP/sAPRIL fusion protein

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Abstract A fusion protein of enhanced green fluorescent protein (EGFP) and soluble domain of human a proliferation-inducing ligand (sAPRIL) was efficiently expressed in *Escherichia coli* BL 21 (DE3). The soluble EGFP/sAPRIL, around 43 kDa, was purified in milligram amounts using metal chelate affinity chromatography and detected with anti-His₆ and anti-hsAPRIL monoclonal antibody. The chimeric protein exhibited similar fluorescence spectra with free EGFP. In vitro, purified EGFP/sAPRIL specifically bound receptor B cell maturation antigen (BCMA) detected by enzyme linked immunosorbent assay (ELISA) and receptors [including heparan sulfate proteoglycan (HSPGs)]-positive cell lines analyzed by fluorescence-activated cell sorting (FACS). Confocal laser microscopy images visibly showed the HSPGs'-dependent binding of EGFP/sAPRIL to NIH-3T3 cell. In addition, the chimera retained the bioactivity to stimulate/co-stimulate proliferation of NIH-3T3 and Jurkat cell/human B cell in vitro. Therefore, the fusion protein shows a readily obtainable source of biologically active sAPRIL which has considerable potential for single-step fluorescence detection assay in the study of APRIL and its receptors.

Keywords Fusion protein · EGFP · APRIL ·
In vitro expression · LSCM

Introduction

A proliferation-inducing ligand (APRIL), also named TALL-2, TRDL-1, and TNFSF-13a is a newly identified

type II membrane protein that belongs to the TNF family (Hahne et al. 1998). It does not exist as a membrane-bound form but is processed intracellularly within the Golgi apparatus by a furin pro-protein convertase before secretion of the biologically active form (Lopez-Fraga et al. 2001). APRIL serves an important role in immunological responses, such as the contribution to plasma cell survival, isotype switching, and T-independent antibody responses (Planelles et al. 2004; Stein et al. 2002; Litinskiy et al. 2002; Castigli et al. 2004). More important, APRIL is extensively expressed in several malignant tumors and promotes growth in several malignant tumor cell lines in vitro and in vivo (Hahne et al. 1998). Two TNF receptor family members, B cell maturation antigen (BCMA) and transmembrane activator and cyclophilin ligand interactor (TACI), bind to APRIL with high affinity (Rennert et al. 2000; Yu et al. 2000; Wu et al. 2000; Marsters et al. 2000). Both receptors are shared with BAFF (also named BLyS, THANK, TALL-1, zTNF-4), another ligand of the TNF family (Mackay et al. 2003). However, neither receptor appears crucial for the tumor-promoting effects of APRIL, because these tumor cells, such as lung carcinoma A549 cell and Jurkat T leukemia cell, lack both TACI and BCMA expression (Rennert et al. 2000), indicating the existence of another unidentified APRIL-specific receptor on these cells surface. Recently, after a long search for a third TNF-R-like receptor, two laboratories demonstrated that heparan sulphate proteoglycans (HSPGs), instead, is the novel 'binding partner' for APRIL, and a basic sequence (KQKQKQ, aa 109–115) near the amino terminus of the mature APRIL protein (out the TNF fold) seems to be responsible for binding HSPGs (Ingold et al. 2005; Hendriks et al. 2005). However, it is unclear what the biological purpose for APRIL binding to HSPG is and how APRIL mediates its enhancement of tumor-cell proliferation solely through interaction with HSPGs. It remains possible that another

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APRIL receptor exists, perhaps, present at extremely low density on these tumor-cells (Dillon et al. 2006). For better research on APRIL, its receptors and their relationship with tumors, in this paper, using enhanced green fluorescent protein (EGFP) (excitation maximum at 488 nm; emission maximum at 507 nm), we constructed a novel fluorescent fusion protein, EGFP/sAPRIL, and demonstrated its application for single-step fluorescence detection assay in the study of APRIL and its receptors.

Materials and methods

Cells

Fresh human peripheral blood mononuclear cells (PBMCs) were purchased from Jiangsu Blood Center, Jiangsu, China. Human B cells were isolated from peripheral blood of normal adult human using anti-CD19 magnetic beads (Mitenyi Biotech) as previously described (Guan et al. 2006a,b). B cells, human embryonic kidney cells 293T, Jurkat T cells, NIH-3T3 fibroblasts cells, and MBL-2 murine lymphoma cells were grown in RPMI 1640 with 10% fetal calf serum (FCS). Burkitt B lymphoma Namalwa cells and lung carcinoma A549 cells were maintained in Dulbecco's modified eagle's medium (DMEM) containing 10% FCS. All media contained antibiotics (100 U/ml penicillin/streptomycin) (Gibco BRL).

RNA isolation, RT-PCR, and sequencing

The total RNA of PBMCs was extracted using the TRIzol reagent (Invitrogen) according to the standard protocol. For reverse transcriptase-polymerase chain reaction (RT-PCR), a first-strand cDNA was synthesized from 1 µg RNA using Reverse Transcriptase XL (AMV) (Takara), according to standard procedures. The primers, GCAGTGCTCACCCA AAAACAGAAG (sense) and TCACAGTTTCACAAACC CCAGGAAG (anti-sense), designed based on the sAPRIL (mature COOH-terminal domain of APRIL, aa 105–250) encoding sequence, were used in the PCR. The amplification condition was as follows: 30 cycles, denaturation 94°C/30 s, annealing 59°C/30 s, extension 72°C/1 min. The endogenously expressed β-actin mRNA was used as an internal control. A pair of β-actin primers, TGTTTGA GACCTTCAACACCC (sense) and AGCACTGTGTTGG CGTACAG (anti-sense) was used to amplify a 528-bp fragment. The same schedule was used for β-actin amplification. The PCR fragments were separated in 2% agarose gels and visualized by ethidium bromide. Then the fragment of sAPRIL was subcloned into pMD18-T vector (Takara), named as pMD18T-sAPRIL, and confirmed by the ABI Prism automated sequencing method.

Construction of expressing vector pET28a-EGFP/sAPRIL

To obtain EGFP/sAPRIL fusion gene first, the method of overlapping polymerase chain reaction (overlap PCR) was used. Four primers for PCR were designed as follows based on the EGFP and the sAPRIL encoding sequence:

- A1 (sense): TCAGGACATATGGTGAGCAAG
- A2 (anti-sense): *GCTGCCACCTCCACCGCTACCGC GCCTCCCTTGTACAGC TC*
- A3 (sense): *GGTGGAGGTGGCAGCGCAGTGCTCA CCCA*
- A4 (anti-sense): ACTGAGGGATCCTCACAGTTTCAA

The primer A2 contained a (Gly₄Ser)₂ linker encoding sequence (boxed). For overlap PCR, the primer A3 and A2 had an overlapping complementary sequence shown in italics, resulting in one fusion cassette containing EGFP, (Gly₄Ser)₂ linker, and sAPRIL encoding sequences. The A1 and the A4 were introduced with *Nde* I and *Bam*H I restriction sites (underlined), respectively, for subsequent cloning. The A1 and the A2 were used to amplify the cDNA sequence of EGFP without the stop codon from the pEGFP-N1 (Clontech). The A3 and the A4 were used to amplify the cDNA sequence of sAPRIL, with pMD18T-sAPRIL above as template. After the first round of PCR using A1/A2 and A3/A4, respectively, gel purification was used for the two products. The second round of PCR was performed using the two resulting PCR products as template and A1/A4 as primers to obtain the cDNA encoding EGFP/(Gly₄Ser)₂/sAPRIL fusion protein. Then, the PCR product was digested by *Nde* I and *Bam*H I and was inserted in frame into pET28a expression vector (Novagen). The recombinant vector was transformed into the competent DH5α cells (Novagen) first, and transformants were screened for Kanamycin (30 mg/ml) resistance. The plasmid from resistance colonies was digested with restriction endonucleases and sequenced to verify its identity and absence of mutation. The recombinant plasmid was named as pET28a-EGFP/sAPRIL (Fig. 1a).

Expression and purification of fusion protein EGFP/sAPRIL

The constructed recombinant plasmid, pET28a-EGFP/sAPRIL, was transformed into competent BL21 (DE3) (Novagen) cells to express fusion protein. The bacteria were cultured in Luria Broth (LB) medium with vigorous shaking (240 rpm) at 37°C to the density of OD₆₀₀≈0.6. For efficiently soluble yield of EGFP/sAPRIL protein, the induction scheme after several tests was established as follows: final isopropyl-beta-D-thiogalactopyranoside (IPTG) concentration, 0.4 mM; induction temperature, 24°C; total induction hours, 6 h; and shaking speed, 200 rpm. After induction, the bacteria were harvested by centrifugation at

10,000×*g* for 5 min at 4°C. The pellet was sonicated on ice for a 10 s-pulse with an intervening 10 s-pause until the cells were completely lysed. The lysate was centrifuged at 12,000×*g* for 30 min at 4°C. Then the supernatant was collected and applied to a nickel column to purify histidine-tagged proteins under nature conditions following the manufacturer's instructions. Potential lipopolysaccharide (LPS) contaminants were removed by incubation with polymyxin B Sepharose resin (Sigma) at 4°C for 30 min. After centrifugation, the purified EGFP/sAPRIL proteins were dialyzed against phosphate-buffered saline (PBS) and sterilized by filtration.

Western blot analysis

The purified fusion proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gel and then blotted to a nitrocellulose membrane (Amersham). The membrane was blocked for 2 h at 37°C with tris-buffered saline Tween-20 (TBST) (25 mM Tris-HCl, 125 mM NaCl, 0.1% Tween 20, pH 8.0) contain-

ing 5% skimmed milk. After washing for five times with TBS, the blots were incubated with anti-His₆ mouse or anti-hsAPRIL rabbit mAb (Sigma) at 37°C for 2 h. After washing three times for 5 min with TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit IgG in TBST containing 5% skimmed milk for 2 h at 37°C. After washing, TMB (Promega) was added, finally, to the corresponding reaction for color development.

Measurement of fluorescence spectra

EGFP/sAPRIL fusion proteins and free EGFP proteins (10 μM) prepared in our laboratory were dialyzed, respectively, with sodium phosphate buffer (20 mM, pH 7.4) overnight at 4°C, and then, their fluorescence spectra were measured using the fluorescence spectrophotometer F-2000 (Hitachi, Tokyo). Emission spectra were measured by excitation wavelength set at 488 nm and excitation spectra were measured by emission wavelength set at 507 nm.

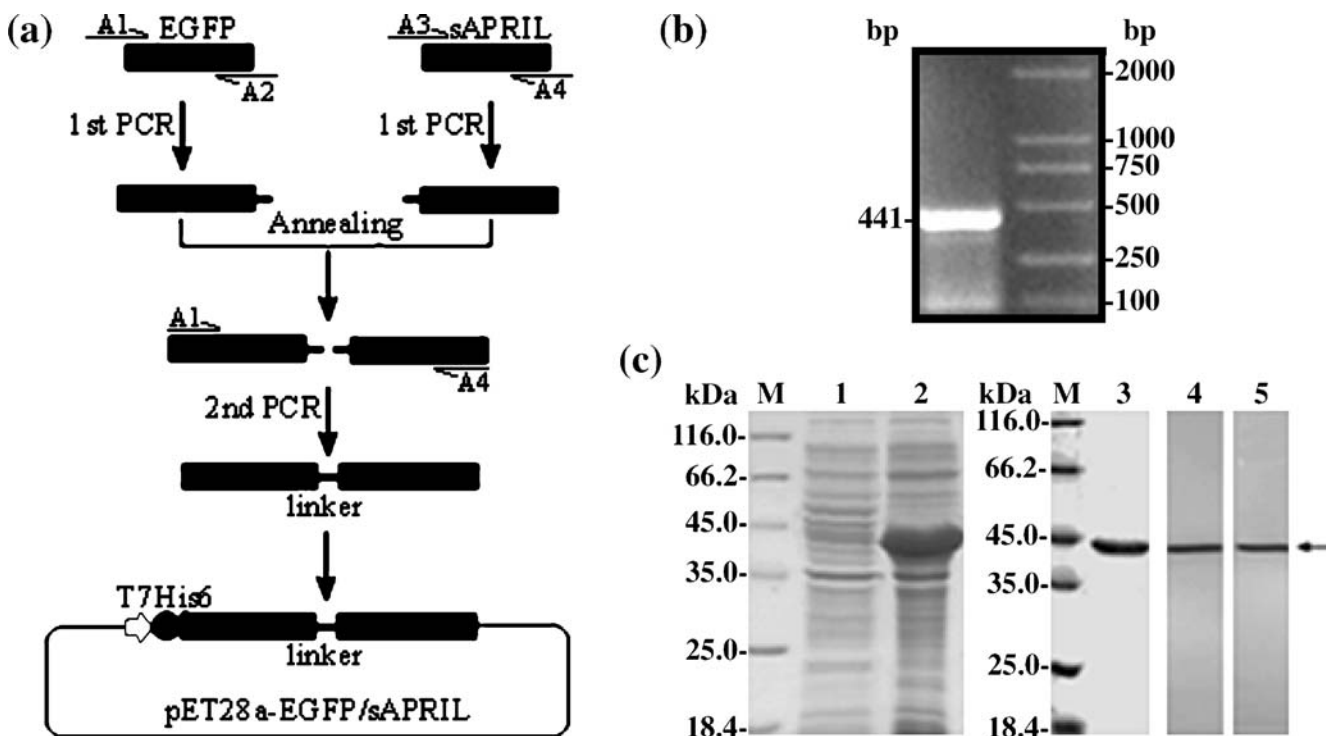


Fig. 1 **a** Schematic representation of the construction procedure of recombinant vector pET28a-EGFP/sAPRIL. Arrows represent the primers used in this experiment. **b** RT-PCR result of sAPRIL (aa 105–250) cDNA amplification from human PBMCs total RNA. The target fragment was 441 bp. Positive control of β -actin amplification and negative controls were not shown. **c** The analysis of EGFP/sAPRIL fusion protein expressed in BL21 (DE3). Lane 1 indicates cell lysates of bacteria transformed with empty pET28a under IPTG

induction. Lane 2 represents cell lysates of bacteria transformed with pET28a-EGFP/sAPRIL under IPTG induction. Lane 3 shows the fusion protein purified by immobilized metal affinity chromatography. Lane 4, 5 show Western blot analysis of purified EGFP/sAPRIL using mAb against His₆ tag and hsAPRIL, respectively. Low molecular weight marker is shown in the left lane. The arrow indicates the location of the recombinant fusion protein

Receptor binding ELISA

EGFP/sAPRIL fusion proteins of 0.01, 0.1, and 1 μg were coated in triplicate onto 96-well microtiter plates (Costar), respectively, overnight at 4°C. After washing with phosphate-buffered saline/Tween (PBST) for 15 min for four times, unoccupied sites on the microplates were blocked with 5% non-fat milk in PBST for 1 h followed by four washes. Then each well was added into BCMA-Fc (1 μg) prepared in our laboratory (Guan et al. 2006a,b) and incubated for 4 h at room temperature followed by four washes. After incubation with HRP-labeled goat anti-human IgG for 1 h at room temperature, the plates were washed for eight times. Peroxidase activity was detected by adding 100 μl /well *o*-phenylenediamine (0.4 mg/ml in 0.05 M phosphate-citrate buffer, pH 5.0) substrate with incubation for 15 min, and reaction was stopped by the addition of 50 μl of 2 M sulfate acid. Reactions were monitored by an automated microtiter plate reader at 490 nm. For controls, lysozyme, His-TRAIL, and free EGFP of 1 μg each were used at the same time.

Flow cytometric analysis

The test cells (2×10^5) in staining buffer (PBS solution with 2% FCS) were incubated with EGFP/sAPRIL (5 μg) or free EGFP (5 μg) for 30 min on ice. After incubation, cells were washed three times with staining buffer. Analysis was performed with FACScalibur (BD biosciences) using a 488-nm laser.

Microscopic observation

NIH-3T3 cells (2×10^5) in staining buffer (PBS solution with 2% FCS) were incubated for 30 min on ice with EGFP/sAPRIL (5 μg) with or without 4 IU/ml heparin. After incubation, cells were washed three times with staining buffer and then resuspended in PBS buffer. Resuspended cells were fixed on microscope slides, and then, images were captured on an LSM 510 confocal microscope using a cooled MicroMax CCD camera. The 488-nm line of krypton–argon laser was used for fluorescent excitation of EGFP. Free EGFP (5 μg) was used as a control protein.

Proliferation assays

The proliferation of Jurkat, NIH-3T3, and human CD19⁺ B cells under different stimulation conditions was determined by incubating 10^5 cells/well in 100 μl medium in triplicate with free EGFP, EGFP/sAPRIL, commercial standard hsAPRIL (R&D), anti-IgM (Sigma), or recombinant hsBAFF prepared in our laboratory (Cao et al. 2005) with indicated concentrations using MTT assay (Mosmann 1983). After incubation at 37°C

for 48 h, 20 μl MTT (5 mg/ml) was added to each well and reacted for 4 h. Then 100 μl of lysis buffer (10% SDS—0.01 M HCl) were added to each well. Plates were incubated for 20 h at 37°C under 5% CO₂, and optical density value (OD) was measured at 570 nm.

Results

Construction of expression vector pET28a-EGFP/sAPRIL

By RT-PCR, we obtained the cDNA sequence (441 bp) encoding the domain of sAPRIL (aa 105–250) (Fig. 1b). By overlap PCR, we obtained EGFP/sAPRIL fusion cDNA with a sequence encoding a flexible linker (Gly₄Ser)₂ between the two domains to avoid structural and functional interference. An effective linker sequence should adopt a flexible extended conformation that provide sufficient distance between receptor binding domains and have minimal hydrophobic or charged character. The cDNA length of EGFP/sAPRIL was 1,182 bp, and the recombinant plasmid containing this cDNA was named pET28a-EGFP/sAPRIL.

In vitro expression and purification of EGFP/sAPRIL fusion protein

The pET28a-EGFP/sAPRIL plasmid was transformed into *Escherichia coli* BL21 (DE3), and the chimera was efficiently solubly expressed at an optimal condition. To simplify the purification process, a His₆ tag was introduced to the N-terminus of the fusion, as this facilitates the easy purification of EGFP/sAPRIL through one step using immobilized metal affinity chromatography. Indeed, when the supernatants of bacteria extract were filtered through the nickel column, the protein fraction with green fluorescence bound to the sorbent. The soluble fusion proteins were finally eluted by 1 M imidazole under nature conditions. According to the SDS-PAGE analysis, after IPTG induction, a 43-kDa band, corresponding to the predicted size of the fusion and absent in BL21 (DE3) transformed with empty pET28a (Fig. 1c, lane 1), was detected in BL21 (DE3) transformed with pET28a-EGFP/sAPRIL (Fig. 1c, lane 2). After one-step purification of soluble fusions, the yield of eluted soluble protein with purity no less than 90% was about 12.5 mg/l bacterial culture (Fig. 1c, lane 3) as assessed by the Bradford method. No attempts were made to remove the impurities by further purification step. The fusions do not tend to precipitate even after long-term storage (data not shown). As is shown in Fig. 1c, purified EGFP/sAPRIL could be detected by anti-His₆ (lane 4) and anti-hsAPRIL mAb (lane 5), indicating a preserved overall conformation of the antigenic moieties recognized by these antibodies within the recombinant fusion protein.

Characterization of EGFP/sAPRIL fusion protein

Fluorescence activity

When green fluorescent protein (GFP) is fused to the N-terminus of overexpressed globular proteins, overexpression in soluble form allows GFP to fold correctly. To make sure the correct folding of the EGFP domain, the purified soluble EGFP/sAPRIL was tested for its fluorescence activity with comparison to free EGFP. As determined by the measurement of fluorescence spectra using the fluorescence spectrophotometer, fluorescence properties of EGFP/sAPRIL were quite similar to free EGFP (excitation maximum at 488 nm; emission maximum at 507 nm), with maximum excitation also at 488 nm and maximum emission also at 507 nm. However, the fluorescence intensity exhibited approximately two times weaker than free EGFP (Fig. 2). It indicates no shift in excitation and emission spectra of EGFP/sAPRIL fusion protein compared to free EGFP. The decrease of fluorescence in chimera might be explained by one mechanism: the fluorophore is formed but the fluorescence is quenched by sAPRIL, which could absorb the inducing or induced radiation.

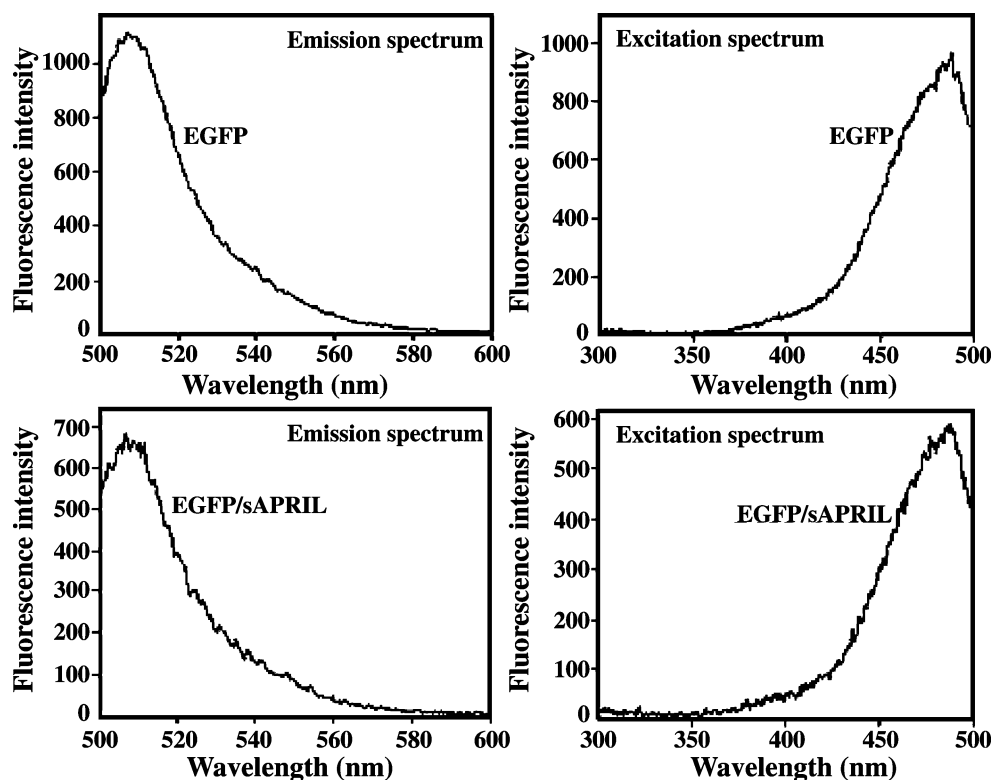
Receptor-binding activity

Soluble, mature APRIL (sAPRIL) is a non-covalent trimer (Wallweber et al. 2004). TNF cytokines bind one elongated

receptor molecule along each of three clefts formed by neighboring monomers of the trimer with ligand trimerization a requisite for receptor binding. To investigate whether the sAPRIL domain fused in EGFP/sAPRIL could form a trimer like parental sAPRIL, and thus, retains the receptor-binding activity, ELISA and FACS assays were used to detect this interaction. As shown in Fig. 3a, compared to control proteins (lysozyme, His-TRAIL, and free EGFP), EGFP/sAPRIL strongly interacts with BCMA-Fc, even with 0.01 μg coated fusions. Next we investigated whether this chimera could interact with membrane-receptors (including HSPGs); flow cytometry was employed to assess this ability by observing whether it could bind receptor-positive cell lines.

Our results showed EGFP/sAPRIL, compared to free EGFP, specifically bound to 293T, Jurkat, NIH-3T3, A549, and Namalwa cell lines, but not to MBL-2 (Fig. 3b), in agreement with the previous observations (Hahne et al. 1998; Rennert et al. 2000; Hendriks et al. 2005). It is known that Namalwa which BAFF specifically binds expresses membrane-receptors BCMA and TACI (Schneider et al. 1999), but 293T, Jurkat, NIH-3T3, and A549 are BCMA- and TACI-negative cell lines which BAFF could not bind (Rennert et al. 2000; Hendriks et al. 2005); it suggests, except its BCMA- and TACI-dependent binding, that the HSPGs-dependent binding of APRIL exists. By FACS analysis, EGFP/sAPRIL likely exhibited the strongest binding efficiency to NIH-3T3 cells, indicating the highest expression of 'binding partners' on this cell surface

Fig. 2 Fluorescence spectra of free EGFP and EGFP/sAPRIL. Emission spectra were measured with 488 nm excitation over the range 500–600 nm. Excitation spectra were measured with 507 nm emission over the range 300–500 nm. Maximum emission wavelengths of free EGFP and EGFP/sAPRIL were both at 507 nm, and maximum excitation wavelengths were both at 488 nm



compared to other binding-positive cell lines. It is noteworthy that the binding activity to cells of EGFP/sAPRIL was retained even for 3 months at 4°C (data not shown). These data suggest that the fusion molecule, EGFP/sAPRIL, could exist as a stable and bioactive trimer in solution.

Laser scanning confocal microscopy (LSCM) utilized in the present study provided a visibly clear image of the distribution of EGFP/sAPRIL which were binding on cell surface by detection of fluorescent signal of EGFP. APRIL binds HSPGs via its N-terminal portion, and this interaction can be blocked by heparin as a heparin-sensitive manner

(Ingold et al. 2005; Hendriks et al. 2005). As shown in Fig. 4, when NIH-3T3 cells were incubated with EGFP/sAPRIL only, fusion proteins could specifically bind to cell surface: the chimera exhibits patches of more intense fluorescence interspersed by areas that are less intense membranous staining pattern, representing the same pattern of ‘binding partners’ distribution on this cell surface. But the binding of EGFP/sAPRIL to this cell was completely inhibited when this chimera was pretreated with heparin in cell culture (data not shown), consistent with the previous observation (Ingold et al. 2005; Hendriks et al. 2005).

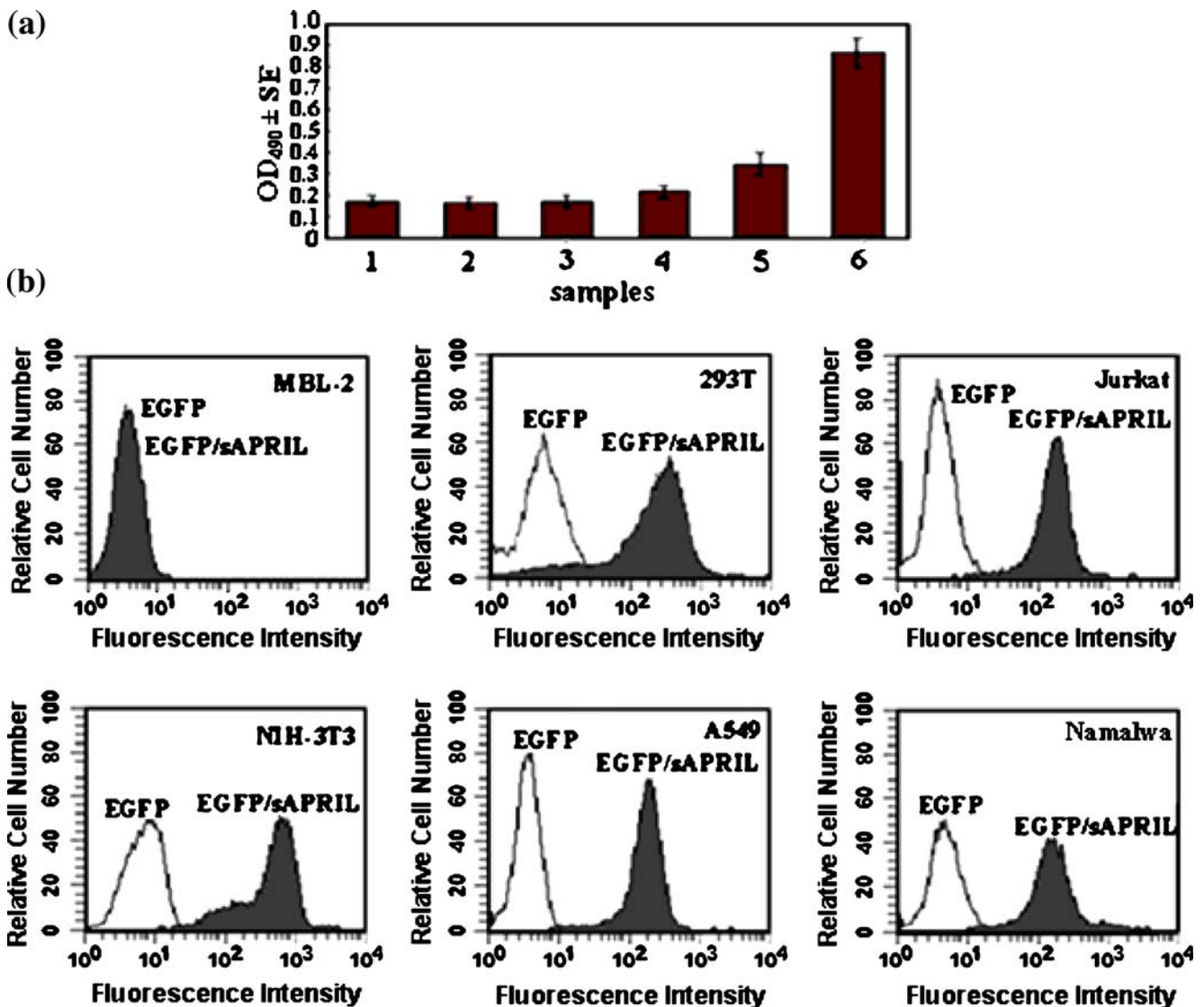


Fig. 3 a EGFP/sAPRIL binds to BCMA-Fc analysed by ELISA. Sample 1, 2, and 3 (protein controls) represent the coated lysozyme, His-TRAIL, and free EGFP of each 1 µg, respectively. Samples 4, 5, and 6 represent the coated EGFP/sAPRIL of 0.01, 0.1, and 1 µg, respectively. All samples were incubated with BCMA-Fc (1 µg) and then detected by incubation with

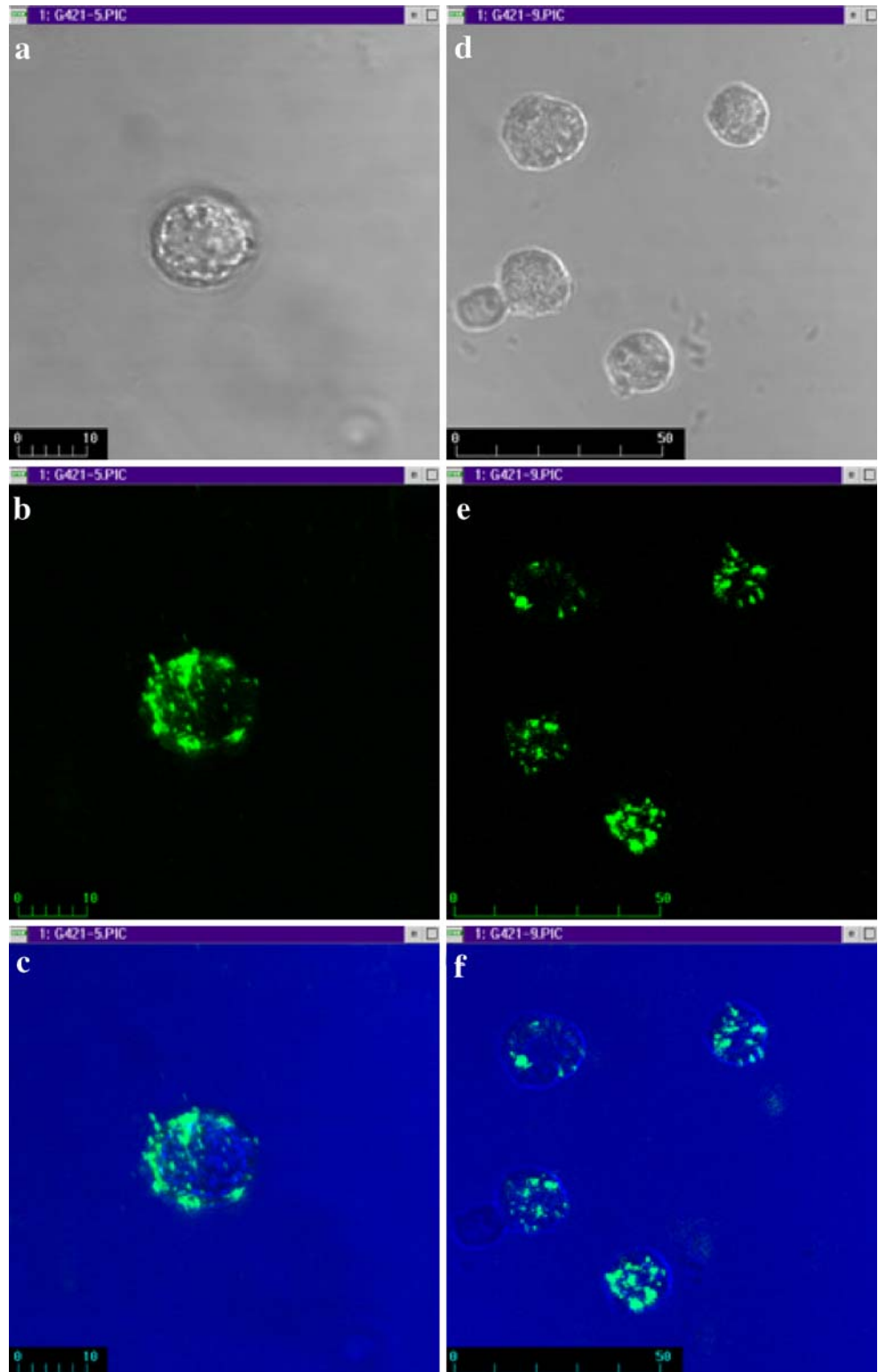
HRP-labeled goat anti-human IgG. Three independent experiments were performed. Values were expressed as mean counts± standard error. b Flow cytometry analysis of EGFP/sAPRIL binding to receptors (including HSPGs)-positive cell lines. *Shaded area*: cells incubated with EGFP/sAPRIL. *Unshaded area*: cells incubated with free EGFP

EGFP/sAPRIL-induced proliferation activity

After confirmation of the fluorescence activity and the receptor-binding activity possessed by EGFP/sAPRIL, we finally investigated its ability to retain proliferation activity

to Jurkat T cell, NIH-3T3 fibroblasts cell, and B cell as parental sAPRIL. As shown in Fig. 5, compared to the controls, EGFP/sAPRIL and commercial sAPRIL both could give a moderate proliferation effect to test cells, with enhancing outgrowth of cells by 20–30%, which is

Fig. 4 Confocal laser microscopy images of EGFP/sAPRIL specific binding to NIH-3T3 cells. Images were captured using a CCD camera with identical settings below the saturation limits. **a** and **d** Images with transmitted light (phase contrast). **b** or **e** Same images as in A or D with EGFP fluorescence (green) following excitation at 488 nm. **c** or **f** Images merged with A/B or D/E. Control protein (free EGFP) did not give any binding signals (data not shown)



consistent with previous observations (Hahne et al. 1998; Rennert et al. 2000; Hendriks et al. 2005), and also, the chimera could co-stimulate human B-cell proliferation in response to anti-IgM, confirming previous studies (Yu et al. 2000; Stein et al. 2002; Castigli et al. 2004).

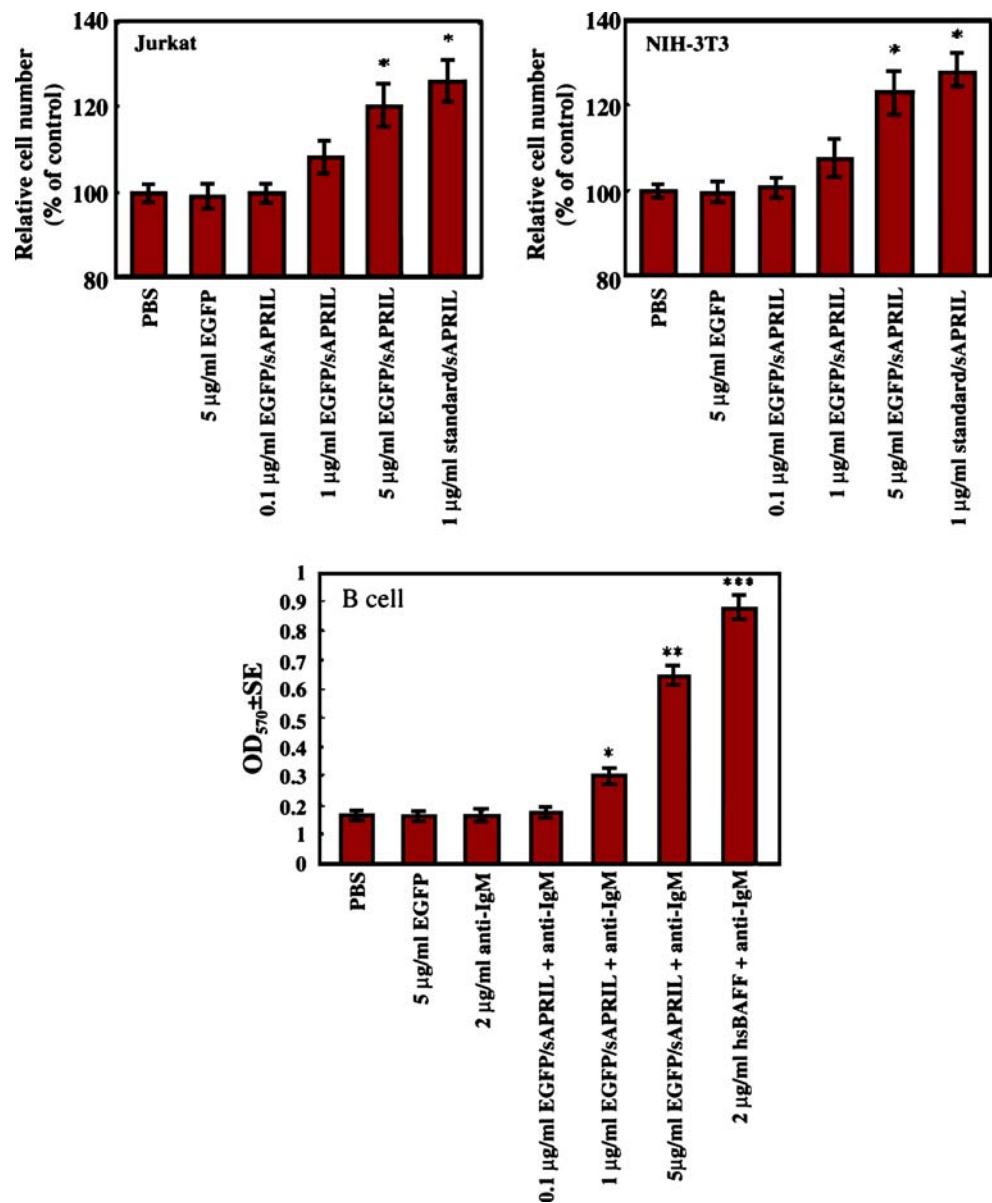
Discussion

It is known that receptors have been studied using radioactive isotopes, ELISA, or functional response in isolated tissue or organ preparations. The disadvantages of these methods, such as radioactive hazards, being cumbersome, time-consuming, and the limitations of studying the molecular dynamics of receptor activation, have hindered the advancement of dynamics research of receptor. GFP is

one of the most popular reporter domains (Tsien 1998). It can be used as a fluorescent live marker when fused with an existing gene, whose product is a naturally fluorescent fusion protein (Chalfie et al. 1994). The advantages of GFP as the reporter domain are its small size (238 amino acid residues), no need for substrates and co-factors, being active as a monomer, stability in a broad range of buffers and temperatures, and easy detection.

The recent development of an enhanced green fluorescence protein (EGFP) genetically engineered for brighter fluorescence has further enlarged the scope of utilization of this protein (Stauber et al. 1998). The use of GFP as a fusion marker permits real-time analysis of receptor dynamics. The emitted fluorescence can be used as a nontoxic marker and detected using fluorescence-activated cell sorting, thus, avoiding any staining procedure, expen-

Fig. 5 In vitro cell proliferation stimulated or co-stimulated by EGFP/sAPRIL. The culture added PBS only was used as a negative control. Proteins were tested in triplicate at the indicated concentrations. The cell culture incubated with free EGFP or anti-IgM was used as a protein control. The culture incubated with standard hsAPRIL (1 $\mu\text{g}/\text{ml}$) or with recombinant hsBAFF and anti-IgM was used as a positive control. hsBAFF and anti-IgM were both used at 2 $\mu\text{g}/\text{ml}$, the concentration previously shown to induce maximal B cell proliferation in the murine and human system (Batten et al. 2000; Cao et al. 2005). The results showed the detection limit of bioactivity of the fusion protein appears to be 1 $\mu\text{g}/\text{ml}$. Three independent experiments were performed. Values were expressed as mean counts \pm standard error. *asterisk* (*) $P < 0.05$, *double asterisks* (**) $P < 0.01$, *triple asterisks* (***) $P < 0.001$ vs control group



sive mRNA analysis or hazardous radiolabeled binding assays. As the detection and tracking of single GFP molecules have been achieved using microscopy techniques, the examination of the chimera at extremely low levels could be also possible. In addition, the confocal scanning image utilized can provide a visibly clearer image of distribution of fluorescent signal on the cell surface. In the present, the potential value of GFP as a fusion marker has been recognized in orphan receptor research. In this study, soluble bioactive fusion protein EGFP/sAPRIL was efficiently produced in *E. coli*, and it exhibited the activities of both domains. EGFP/sAPRIL had similar fluorescence activity as free EGFP. It could not only specifically bind to receptor BCMA and receptor-positive cell lines, but also stimulate/co-stimulate proliferation of NIH-3T3 and Jurkat cell/human B cell in vitro. In summary, our studies indicated that this bifunctional fusion protein holds great potential as an enhanced molecule in analysis of the site and time course of receptor expression and to relate receptor dynamics to therapeutic outcome.

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References

- Batten M, Groom J, Cachero TG, Qian F, Schneider P, Tschopp J, Browning JL, Mackay F (2000) BAFF mediates survival of peripheral immature B lymphocytes. *J Exp Med* 192:1453–1466
- Cao P, Mei JJ, Diao ZY, Zhang SQ (2005) Expression, refolding and characterization of human soluble BAFF synthesized in *Escherichia coli*. *Protein Expr Purif* 41:199–206
- Castigli E, Scott S, Dedeoglu F, Bryce P, Jabara H, Bhan AK, Mizoguchi E, Geha RS (2004) Impaired IgA class switching in APRIL-deficient mice. *Proc Natl Acad Sci USA* 101:3903–3908
- Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC (1994) Green fluorescent protein as a marker for gene expression. *Science* 263:802–805
- Dillon SR, Gross JA, Ansell SM, Novak AJ (2006) An APRIL to remember: novel TNF ligands as therapeutic targets. *Nat Rev Drug Discov* 5:235–246
- Guan ZB, Cao P, Ye JL, Zhang SQ (2006a) Cloning, soluble expression and characterization of human sBCMA. *Sheng Wu Gong Cheng Xue Bao* 22:46–51, Chinese
- Guan ZB, Ye JL, Dan WB, Yao WJ, Zhang SQ (2006b) Cloning, expression and bioactivity of duck BAFF. *Mol Immunol* (in press). PMID 16828163
- Hahne M, Kataoka T, Schroter M, Hofmann K, Irmeler M, Bodmer JL, Schneider P, Bornand T, Holler N, French LE, Sordat B, Rimoldi D, Tschopp J (1998) APRIL, a new ligand of the tumor necrosis factor family, stimulates tumor cell growth. *J Exp Med* 188:1185–1190
- Hendriks J, Planelles L, Jong-Odding JD, Hardenberg G, Pals ST, Hahne M, Spaargaren M, Medema JP (2005) Heparan sulfate proteoglycan binding promotes APRIL-induced tumor cell proliferation. *Cell Death Differ* 12:637–648
- Ingold K, Zumsteg A, Tardivel A, Huard B, Steiner Q, Cachero TG, Qiang F, Gorelik L, Kalled SL, Acha-Orbea H, Rennert PD, Tschopp J, Schneider P (2005) Identification of proteoglycans as the APRIL-specific binding partners. *J Exp Med* 201:1375–1383
- Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, Casali P, Cerutti A (2002) DCs induce CD40-independent immunoglobulin class switching through BLYS and APRIL. *Nat Immunol* 3:822–829
- Lopez-Fraga M, Fernandez R, Albar JP, Hahne M (2001) Biologically active APRIL is secreted following intracellular processing in the Golgi apparatus by furin convertase. *EMBO Rep* 2:945–951
- Mackay F, Schneider P, Rennert P, Browning J (2003) BAFF AND APRIL: a tutorial on B cell survival. *Annu Rev Immunol* 21:231–264
- Marsters SA, Yan M, Pitti RM, Haas PE, Dixit VM, Ashkenazi A (2000) Interaction of the TNF homologues BLYS and APRIL with the TNF receptor homologues BCMA and TACI. *Curr Biol* 10:785–788
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55–63
- Planelles L, Carvalho-Pinto CE, Hardenberg G, Smaniotto S, Savino W, Gomez-Caro R, Alvarez-Mon M, de Jong J, Eldering E, Martinez AC, Medema JP, Hahne M (2004) APRIL promotes B-1 cell-associated neoplasia. *Cancer Cell* 6:399–408
- Rennert P, Schneider P, Cachero TG, Thompson J, Trabach L, Hertig S, Holler N, Qian F, Mullen C, Strauch K, Browning JL, Ambrose C, Tschopp J (2000) A soluble form of B cell maturation antigen, a receptor for the tumor necrosis factor family member APRIL, inhibits tumor cell growth. *J Exp Med* 192:1677–1684
- Schneider P, MacKay F, Steiner V, Hofmann K, Bodmer JL, Holler N, Ambrose C, Lawton P, Bixler S, Acha-Orbea H, Valmori D, Romero P, Werner-Favre C, Zubler RH, Browning JL, Tschopp J (1999) BAFF, a novel ligand of tumor necrosis factor family, stimulates B cell growth. *J Exp Med* 189:1747–1756
- Stauber RH, Horie K, Carney P, Hudson EA, Tarasova NI, Gaitanaris GA, Pavlakis GN (1998) Development and applications of enhanced green fluorescent protein mutants. *Biotechniques* 24:462–471
- Stein JV, Lopez-Fraga M, Elustondo FA, Carvalho-Pinto CE, Rodriguez D, Gomez-Caro R, De Jong J, Martinez AC, Medema JP, Hahne M (2002) APRIL modulates B and T cell immunity. *J Clin Invest* 109:1587–1598
- Tsien R (1998) The green fluorescent protein. *Annu Rev Biochem* 67:509–544
- Wallweber JA, Compaan DM, Starovasnik MA, Hymowitz SG (2004) The crystal structure of a proliferation-inducing ligand, APRIL. *J Mol Biol* 343:283–290
- Wu Y, Bressette D, Carrell JA, Kaufman T, Feng P, Taylor K, Gan Y, Cho YH, Garcia AD, Gollatz E, Dimke D, LaFleur D, Migone TS, Nardelli B, Wei P, Ruben SM, Ullrich SJ, Olsen HS, Kanakaraj P, Moore PA, Baker KP (2000) Tumor necrosis factor (TNF) receptor superfamily member TACI is a high affinity receptor for TNF family members APRIL and BLYS. *J Biol Chem* 275:35478–35485
- Yu G, Boone T, Delaney J, Hawkins N, Kelley M, Ramakrishnan M, McCabe S, Qiu WR, Kornuc M, Xia XZ, Guo J, Stolina M, Boyle WJ, Sarosi I, Hsu H, Senaldi G, Theill LE (2000) APRIL and TALL-1 and receptors BCMA and TACI: system for regulating humoral immunity. *Nat Immunol* 1:252–256