J. Biochem. **142,** 471–480 (2007)
doi:10.1093/jb/mvm160

Functional and Stable Expression of Recombinant Human FOXP3 in Bacterial Cells and Development of Antigen-specific Monoclonal Antibodies

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Received June 20, 2007; accepted July 13, 2007; published online August 30, 2007

FOXP3 is a member of the forkhead/winged-helix family of transcriptional regulators which plays a key role in $CD4^+CD25^+$ regulatory T-cell (Tregs) function and represents a specific marker for these cells. In order to understand the functional role of FOXP3 and identify Tregs both in normal development and relevant diseases, protein–DNA and protein–protein interaction studies involving this factor are essentially required. Such investigations would be facilitated by the availability of significant amounts of purified FOXP3 protein and specific McAb against FOXP3. Here, we report the purification of human FOXP3 (HFOXP3) expressed in *Escherichia coli* as a soluble and functional glutathione-S-transferase (GST) fusion protein. Through a single purification procedure, 4 mg of GST-HFOXP3 recombinant protein was obtained per litre of bacterial culture. The biological activity of the recombinant protein was verified by EMSA assay. The yield of folded FOXP3 in the purified GST-FOXP3 was determined by reverse phase HPLC. Besides, we generated and obtained two specific monoclonal antibodies by immunizing BALB/c mouse with the purified GST-HFOXP3 protein. The resulting HFOXP3 protein and anti-HFOXP3 McAbs might provide a useful tool in studying Tregs development and immune regulations.

Key words: FOXP3, monoclonal antibody, protein expression, purification, Tregs.

Abbreviations: EMSA, electrophoretic mobility-shift assay; FOXP3, forkhead box P3; human FOXP3, HFOXP3; McAb, monoclonal antibody; Tregs, regulatory T cells.

FOXP3 is a member of the forkhead/winged-helix family of transcriptional regulators. The gene was first described as JM2 when its mutation was identified in an X-linked autoimmune and allergic disregulation syndrome (XLAAD) (*1*). In mice, FOXP3 mutations is responsible for the scurfy mouse phenotype and the human FOXP3 mutation cause X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome (IPEX) (*2–4*). Initial studies of scurfy mice indicated that $CD4^+$ T cells from these mice were hyper-responsive to T cell antigen receptor (TCR) stimulation and produced excessive amounts of cytokines (*5*, *6*). The absence of FOXP3 in both mice and humans thus causes a fatal immune proliferation disease, as a consequence of chronic T-cell activation (*7*). More recently, FOXP3 has been identified as a master regulatory gene for cell-lineage commitment or developmental differentiation of $CD4^+CD25^+$ regulatory T cells (Tregs) (*8–10*). Ectopic expression of FOXP3 *in vitro* and *in vivo* is sufficient to convert naive murine $CD4^+$ T cells to Tregs. Furthermore, transfer of $CD4^+CD25^+$ T cells can delay the progression of autoimmune diseases in FOXP3-deficient mice (*8–10*). Thus, FOXP3 appears to represent an important marker of Treg population.

Although the association of FOXP3 with Tregs provides a significant step toward our understanding of the function of FOXP3, little is known about the biochemical mechanisms by which FOXP3 acts in T cells. A common feature of FOX family proteins is the FKH domain, which has been shown to be necessary and sufficient for DNA binding. DNA-binding analysis from a number of the family proteins have defined a core DNA sequence (5′-A(A/T)TRTT(G/T)R-3′, where R = pyrimidine) surrounded by less conserved sequences. Experimentally, two canonical FKH-binding sequences [from the transthyretin promoter (TTR-S) and the immunoglobulin variable regions V1 promoter (V1P)] have been used as templates to analyse the DNA-binding properties of FOX proteins (*11–13*). Similar to other members of the FOX family, FOXP3 can bind to each of these FKH-binding sites (*14*). Cellular target genes of transcription factors are

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well understood in the context of interacting partner proteins which form transcriptional complexes assembled on gene promoters or enhancers. As studies with other transcription factors have shown, approaches are available which utilize recombinant protein to functionally dissect their role by identifying or confirming target genes and/or interacting partner proteins (*15–17*). Such studies require significant amounts of HFOXP3 protein with high purity.

Treg cell represents a major lymphocyte population engaged in the dominant control of self-reactive T responses and maintenance of tolerance. It down-regulates immune responses in various inflammatory circumstances and ultimately assure peripheral T-cell tolerance. In patients with autoimmune diseases, reduced levels of circulating Tregs were described, specifically in individuals with inflammatory bowel disease (*18*), multiple sclerosis (*19*), allograft rejection (*20*), autoimmune liver disease (*21*), and systemic lupus erythematosus (*22*). Lower levels of circulating Tregs also correlate with a higher disease activity or poorer prognosis (*23*). However, high Treg numbers enable cancer cells to evade the host immune response. Many clinical studies have demonstrated increased number of Tregs in humans with a diverse range of malignancies such as gastric carcinoma (*24*), ovarian carcinoma (*25*), lung carcinoma (*26*), colorectal carcinoma (*27*), and hepatocellular carcinoma (*28*). There are a larger amount of Tregs in the peripheral blood, tumour-infiltrating lymphocytes (TILs) or tumour-draining lymph nodes of cancer patients (*24*). The frequencies and function of Tregs are important because increased numbers might favour tumour growth and influence the course of the disease (*29*). The high prevalence of Tregs seems to be a marker of poor prognosis, while the depletion of Tregs prolongs the survival of mice with many different tumours (*30*). The transcription factor FOXP3 appears to be a specific marker of Treg population. Thus, the identification of FOXP3 means the direct identification of Tregs for diagnosis. So, it is important to develop the specific McAb against FOXP3 which can be used in a variety of applications, including western blotting, flow cytometry and cytochemistry.

We therefore cloned human full-length FOXP3 cDNA into a pGEX expression vector and established a high effective purification protocol in order to obtain functional HFOXP3 fusion protein in large quantities and in high purity. We also generated two specific anti-HFOXP3 McAbs by immunizing BALB/c mouse with the recombinant GST-HFOXP3 protein in order to identify Tregs in both normal tissues and relevant diseases. Finally, we tested the function of recombinant HFOXP3 protein and anti-HFOXP3 McAb.

MATERIALS AND METHODS

Reagents, Strains, Vectors—Escherichia coli TG1 and BL21 (DE3) were used as the cloning and expression host cells, respectively. Fusion expression vector pGEX-4T-1 was purchased from Amersham (Beijing, China). The restriction enzymes EcoR I and Xho I, T4 DNA ligase and Pfu DNA polymerase were purchased from Takara (Takara, Dalian, China). The gel extraction kit was purchased from Qiagen (Valencia, CA, USA). Ampicillin, phenylmethylsulphonyl fluoride (PMSF), isopropyl-β-D-thiogalactoside (IPTG), Triton X-100, reduced glutathione (GSH), and cell culture reagents were purchased from Sangon (Shanghai, China). Chromatographic resins and glutathione-Sepharose 4B were obtained from Pharmacia Biotech. All other chemical reagents were of analytical grade.

Cloning of Human Foxp3 cDNA and Plasmid Construction—Total RNA was extracted from human peripheral blood mononuclear cells with the total RNA Isolation kit (Takara, Dalian, China). Synthetic oligonucleotide primers (5′ primer, 5′-AA **GAA TTC** ATG CCC AAC CCC AGG CCT-3′; and 3′ primer, 5′- TA **CTC GAG** *TCA* AGG GCA GGG ATT G-3′) were designed based on the reported human Foxp3 cDNA sequence. The incorporated 5′ EcoR I and 3′ Xho I restriction sites are shown in bold and the stop codon in italics. By using the Advantage one-step RT-PCR kit (Clontech, CA, USA), the coding region of human foxp3 cDNA was amplified from the total RNA of human peripheral blood mononuclear cells. The resulting PCR product was gel purified and cloned into *E. coli* expression vector pGEX-4T-1 using EcoR I and Xho I restriction sites. The recombinant plasmid was transformed into *E. coli* TG1 and the transformants were confirmed by DNA sequencing.

B.Human FOXP3 Expression and Purification—The pGEX-HFOXP3 plasmid was transformed into *E. coli* BL21 (DE3) cells. Transformants were grown in LB medium (tryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l and ampicillin 100 μg/ml). The high-expression clones were obtained according to the results of SDS–PAGE analysis and cultured in LB medium overnight at 37°C for small-scale culture. The overnight culture (100 ml) was then inoculated into 900 ml of the fresh medium described above at 37°C until the optical density (A_{600}) reached ~1.0–1.2, and protein expression was induced by adding IPTG to a final concentration 0.5 mM. After induction for 3 h, the cells were separated from the culture medium by centrifugation at 8,000*g* for 15 min, and the cellular pellet was then resuspended in 20 mM sodium phosphate buffer, pH 7.2, containing 150 mM NaCl (PBS) and the cells were lysed thoroughly by ultrasonication at 4°C, and then PMSF (100 mM in absolute alcohol) was added to a final concentration of 1 mM to inhibit protease activity. Triton X-100 was then added to a final concentration of 1% (W/V). Bacterial debris was then removed by centrifugation (14,000*g*, 15 min, 4°C) and the supernatant collected for further purification. The bacterial proteins in the supernatant were analysed by SDS–PAGE. The GST-HFOXP3 fusion protein was purified by glutathione affinity chromatography. A 10 ml Pharmacia glutathione-Sepharose 4B affinity column was equilibrated in phosphate-buffered saline (PBS; 150 mM NaCl, 20 mM Na_2HPO_4, pH 7.2). The clarified cell lysate was subsequently loaded onto the column at 0.5 ml/min. The column was then washed with 10 bed volumes of PBS. The bound protein was subsequently eluted with 10 mM reduced glutathione in 50 mM Tris buffer (pH 8.0) and concentrated by Centricon Microconcentrators (Millipore, Billerica, MA) with 50 kDa molecular weight cut-off. The yield of the

GST-HFOXP3 fusion proteins was estimated by measuring their absorbance at 280 nm. Purified GST-HFOXP3 was analyzed by SDS–PAGE under reducing conditions using 10% gels and the Tris–glycine system of Laemmli. The separated proteins were stained with Coomassie brilliant blue R-250.

Electrophoretic Mobility-Shift Assays—EMSAs were done to assess the activation of the recombinant HFOXP3. Biotin end-labelled double-stranded oligonucleotides TTR-S (5′- biotin-TCG AGT TGA CTA AGT CAA TAA TCA GAA TCA G-3′) and V1P (5′- biotin-TCA AAA ATA TTG AAG TGT TAT CAC ATA CAC-3′) containing consensus FKH-binding sites were used as probes. The binding reactions contained 2 μg of GST-HFOXP3, buffer (10 × Binding Buffer, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% NP-40, and 2.5% glycerol), 1 μg of poly (dI-dC), and 2 nM of biotin-labelled DNA. The reactions were incubated at 25°C for 20 min. The competition reactions were performed by adding 100-fold excess unlabelled double-stranded oligonucleotide to the reaction mixture. The shift assay was performed by adding 2 μg of anti-HFOXP3 McAb (eBioscience, San Diego, CA, USA). And also the commercial anti-HFOXP3 McAb were used as controls in the following experiments for its wide application. The reactions were electrophoresed on a 6% precasted Tris-Borate-EDTA gel (Invitrogen, San Diego, CA, USA) at 100 V for 1 h in a 100 mM Tris–Borate-EDTA buffer. The reactions were transferred to a nylon membrane and the membrane was UV cross-linked. The biotin-labelled DNA was detected with LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce, IL, USA).

The Yield of Folded HFOXP3 in the Purified GST-HFOXP3—Purified GST-HFOXP3 was analysed by reverse phase HPLC on a preparative C4 column. After sample injection, elution was performed with a linear gradient of acetonitrile from 0 to 60% over 60 min. Appropriate fractions were pooled and analyzed by EMSA assay as described above. The amount of proteins in different peaks was determined by integrating the peak area.

Production of HFOXP3-Specific McAb—Female BALB/c mice (~6–8 weeks old) were subcutaneously immunized with ~50–100 μg GST-HFOXP3 fusion protein emulsified in Freund's complete adjuvant (Sigma, St Louis, MO, USA) initially. Fourteen days later, each animal received booster immunizations with the same dose emulsified in Freund's incomplete adjuvant (Sigma, St Louis, MO, USA) three times at 2-week intervals. A 100 μg booster of the recombinant GST-HFOXP3 protein was injected into the tail vein, and cell fusion was carried out 3 days later according to the modified method of Kohler and Milstein (*31*). Parental cell line used for fusion is murine non-secretary myeloma cell line NS-1. The fused cells were suspended in hypoxanthine-aminopterin-thymidine (HAT) medium (Gibco, Grand Island, NY) containing 10^{-4} M hypoxanthine, 10^{-7} M aminopterin, 10^{-8} M thymidine, 20% fetal calf serum (FCS), and antibiotics and incubated at 37°C in 5% CO_2 incubator. After ~10–15 days, the clones secreting anti-HFOXP3 antibodies were screened with ELISA. Ninety-six-well microtitre plates were coated with 100 μl of a 5 μg/ml solution of either recombinant GST-HFOXP3 or GST-TIP30 (an unrelated GST fusion protein as control) in coating buffer (Na_2CO_3, $NaHCO_3$; pH 9.6) respectively for ELISA. Selected clones were sub-cloned four times by limited dilution. The resulting specific antibodies were developed and prepared by producing ascities in pristine injected BALB/c mice. Isotypes of McAbs were determined following the manufacturer's instructions of isotyping kit (Sigma, St Louis, MO, USA).

Western Blotting Analysis—Purified GST-HFOXP3, native HFOXP3 of human PBMC and native MFOXP3 of mouse lymphocytes were analysed by SDS–PAGE under reducing conditions using 10% gels and the Tris–glycine system of Laemmli. The separated proteins were transferred to nitrocellulose membrane for western blotting analysis. After being blocked with 5% non-fat milk in PBST (PBS containing 0.05% Tween-20), the membrane was washed three times with PBST for 10 min and incubated further with hybridoma culture supernatant for 1 h at room temperature. The membrane was washed three times with PBST and incubated with a 1:2000 dilution of horseradish peroxidase (HRP)-labelled goat anti-mouse IgG (US Biological, MA, USA) and visualized with an enhanced chemiluminescence procedure.

Flow Cytometry—Human peripheral blood was drawn from healthy adult volunteers. Heparinized blood was diluted 1/1 v/v with PBS. PBMCs were prepared by Ficoll gradient centrifugation. The buffy coat containing PBMC was harvested, contaminating RBCs lysed by incubating in ACK lysing buffer solution (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.4), and washed twice in cold PBS. Isolated PBMC from peripheral blood were labelled with Cy-Chrome-conjugated anti-human CD4 (BD Pharmingen), PE-conjugated anti-human CD25 (BD Pharmingen), mouse anti-human FOXP3 were obtained and commercial product from eBioscience (San Diego, CA, USA), and FITC-conjugated goat anti-mouse IgG. (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The whole process of staining follows the protocol of the FOXP3 Staining Buffer Set (eBioscience, San Diego, CA, USA). Labelled cells were determined by using FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and the results were analysed using the FlowJo software (Tree Star, Inc., OR, USA).

Production of HFOXP3 Transfectants and Immunocytochemistry—To confirm that the anti-HFOXP3 McAbs we obtained were specifically reactive with the native HFOXP3 protein *in situ*, their reactivity was tested on CHO cells expressing HFOXP3 using immunocytochemistry. The whole length of HFOXP3 DNA with EcoR I and Xho I restriction sites in 5′ and 3′ region was cut down from pGEX-4T-1 and cloned into eukaryotic expression vector pcDNA3. Plasmid pcDNA3-HFOXP3 was prepared for transfection using the Plasmid Midi Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). CHO cells were transfected with pcDNA3-HFOXP3 using Lipofectamine™ 2000 reagent, following the instructions described by the manufacturer (Invitrogen, San Diego, CA, USA). Approximately 48 h post-transfection, the immunocytochemistry was done following the instructions from

the website of IHCWorld online with some modifications. Briefly, place glass coverslips in 6-well plastic petri dishes with about 10^5 trypsinization cells and allow the cells to grow to confluence. Wash the cells, fix cells in ice cold acetone for 10 min and rinse 3 changes in PBS. Incubate the slides with 0.25% Triton X-100 in PBS for 10 min to permeabilize the membranes and rinse in PBS. Then block endogenous peroxidase by incubating in 3% H_2O_2 in PBS for about 20 min and rinse. After blocking with 5% normal goat serum in PBS for 1 h, about 100 μl of mouse anti-HFOXP3 McAbs diluted to the desired concentration in 1% BSA/PBS was added to each coverslip and incubated for 1 h. Wash the coverslips and incubate each coverslip with 100 μl of horseradish peroxidase (HRP)-labelled goat anti-mouse IgG (US Biological, MA, USA) diluted in the desired concentration in 1% BSA/PBS for 30 min. The peroxidase was then developed by the diaminobenzidine (DAB) and detected using the DAKO EnVision method as directed by the manufacturer (Dako, Carinteris, CA, USA).

Detection of HFOXP3 Using Anti-HFOXP3 McAb Sepharose—We only chose the McAb 4D8 to test whether it could be used for immunoaffinity chromatography. The McAb was linked via amine groups to HiTrap NHS-activated 1 ml column (Amersham Pharmacia). The coupling was carried out according to the manufacturer's recommendation. The separation was carried out with the supplied luer adaptor. The column was washed with 0.1 mol/l acetic acid (pH 4.0) and then equilibrated with 0.1 mol/l Tris–HCl buffer (pH 8.3). Through buffer exchange with a centrifugal concentrator (50 kDa cut-off), the buffer was replaced with 0.1 mol/l Tris–HCl buffer (pH 8.3) and 15 ml of purified GST-HFOXP3 fusion protein was then concentrated down to 1 ml. The concentrated fraction was applied to the column and then washed with 15 ml of 25 mM Tris–HCl (pH 8.7) containing 0.3 mol/l NaCl. The protein was eluted with 6 ml of 0.2 mol/l Gly-HCl (pH 2.8). Through a 50-kDa cut-off Centricon filter the sample was then concentrated down to 500 μl. The results were analyzed by SDS–PAGE under reducing conditions using 10% gels and the Tris–glycine system of Laemmli.

RESULTS AND DISCUSSION

Construction and Expression of the Recombinant Vector—The full-length wild-type human Foxp3 amplified by RT-PCR from total RNA extracted from human peripheral blood mononuclear cells was 1.3 kb as designed, which contained the entire protein-coding region of human FOXP3 and restriction endonuclease sites. The fragment was then cloned into the vector pGEX-4T-1 to create pGEX-HFOXP3. The constructed recombinant plasmid pGEX-HFOXP3 for expression of the GST-HFOXP3 fusion protein is shown in (Fig. 1), which contains the human FOXP3 gene (1296 bp, 431 amino acids) fused at its 5′ end to the gene coding for *Spiraea japonica* GST (Sj-GST, 654 bp, 218 amino acids). The two genes were linked by 33 nucleotides which transcribed and translated a sequence of 11 amino acid residues (Ser-Asp-Leu-Val-Pro-Arg-Gly-Ser-Pro-Glu-Phe).

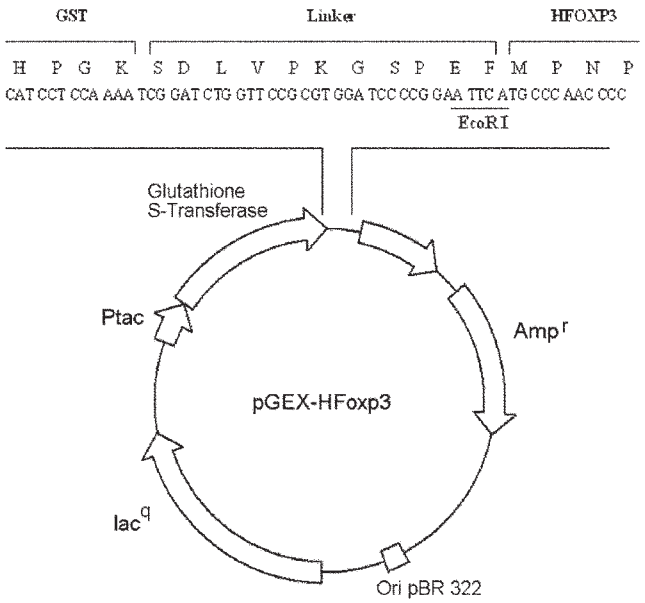

Fig. 1. **The map of plasmid pGEX-HFOXP3.** The EcoR I recognition sequence is shown underlined.

TG1 competent cells were transformed and screened in LB medium containing ampicillin (100 mg/l). The plasmids extracted from positive clones were further investigated by the restriction endonuclease cutting and DNA sequencing. The strain BL21 (DE3) was selected as the host strain, which was deficient in the Ion protease and lacked the ompT outer membrane protease that degraded proteins during the process of purification, so that proteins were more stable in the host. The recombinant protein production was carried out under conditions whereby degradation of the fusion proteins was minimized. This entailed growing the bacterial broths to an OD_{600} of 1.2 before adding 0.5 mM IPTG, and culturing the cells for an additional 3 h. Incubation for longer periods did not result in a higher yield of protein expression, while induction at lower cell densities resulted in a poor yield of GST-HFOXP3 (data not shown). To establish that the GST-HFOXP3 fusion proteins had been expressed in a soluble form in *E. coli*, harvested cells were ultrosonicated in PBS solution on ice, centrifuged, and separated into supernatant and pellet fractions which were analyzed by SDS–PAGE. The major proportion of target protein was found in the supernatant accounting for most of the total soluble cellular proteins as estimated by densitometric analyses (Fig. 2, lane 3), while partial target protein appeared as inclusion bodies in the pellet (Fig. 2, lane 4). Thus, GST-HFOXP3, which has apparent molecular weights of 74 kDa, was mainly expressed as soluble proteins in *E. coli*.

Purification of Recombinant Protein—The expression of HFOXP3 proteins as GST fusions allowed for their convenient purification from other soluble bacterial proteins by glutathione-Sepharose 4B affinity chromatography. Supernatant samples were initially purified at room temperature and the recombinant GST-HFOXP3

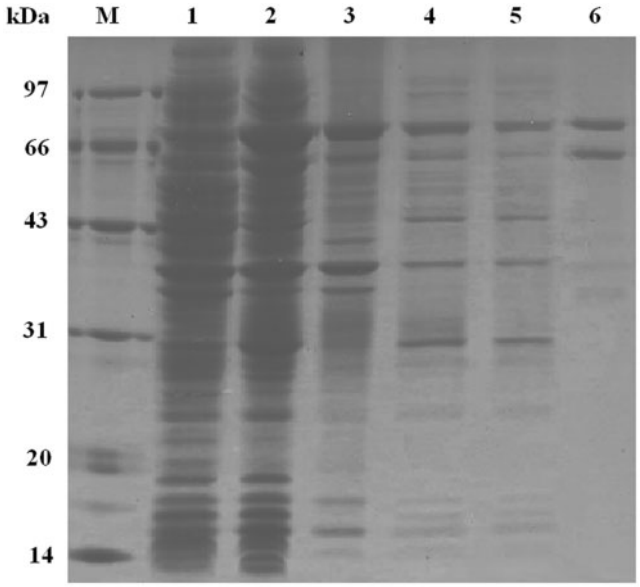

Fig. 2. **Production and purification of GST-HFOXP3 fusion protein.** Proteins were analyzed on 10% SDS–PAGE gel and Coomassie brilliant blue R-250 stained. Lane M, molecular weight markers; lane 1, total protein of *E. coli* before IPTG induction; lane 2, total protein of *E. coli* after incubation in the presence of 0.5 mM IPTG for 3 h; lane 3, soluble fraction from the induced *E. coli* with major proportion of GST-HFOXP3 fusion protein; lane 4, precipitated fraction of the induced cells with partial GST-HFOXP3 fusion protein; lane 5, flow-through fraction from affinity column; lane 6, elute from affinity column at room temperature.

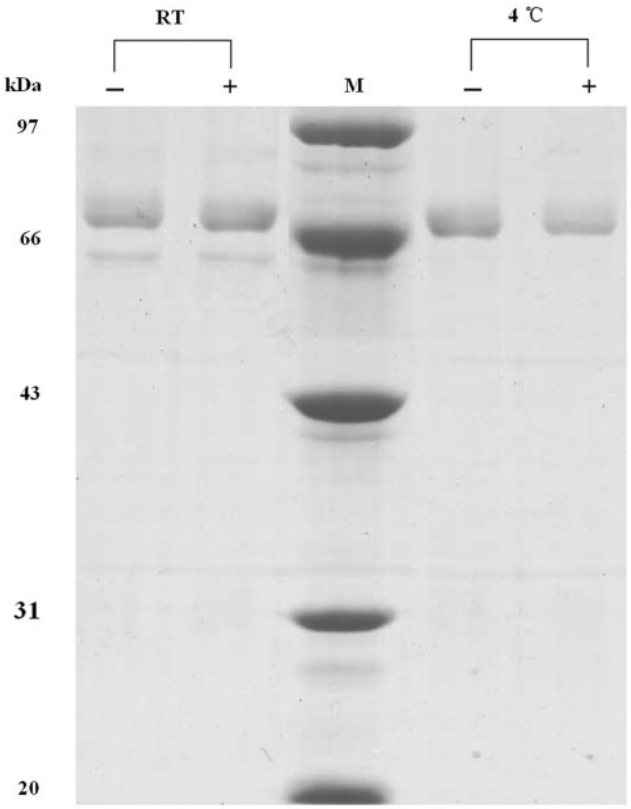

Fig. 3. **Purification of GST-HFOXP3 fusion protein by affinity chromatography using glutathione-Sepharose.** Protein purified at room temperature (RT) or 4°C with or without prior addition of 0.1% Triton X-100 (+/−) to cells, was loaded onto a 10% SDS–PAGE gel and stained with Coomassie brilliant blue R-250. After purification at 4°C, GST-HFOXP3 was shown as a unique band migrating at the apparent molecular weight of 74 kDa.

protein eluted from the beads was then checked on a SDS–PAGE gel stained with Coomassie brilliant blue R-250. Lane 6 in Fig. 2 shows that two major proteins were co-purified. One of them was characterized as a 74 kDa protein corresponding to the GST-HFOXP3 fusion protein. The second was about a 55 kDa protein that might be partially degraded or truncated fusion protein. This may indicate that GST-HFOXP3 fusion proteins are unstable. Therefore, the recombinant protein purification was carried out at low temperature (4°C) and the result is only about one single undegraded band of ~74 kDa, albeit of reduced overall yield (Fig. 3, lanes 4 and 5) (*32*). Thus, under conditions which minimize protein degradation we observed that intact GST-HFOXP3 could be produced with high level of purity, and at a final yield of ~4 mg/l of bacterial culture (Table 1). Recently, Hiraoka *et al*. (*33*) reported successful expression of human FOXP3 protein as Trx fusion protein in *E.coli*. However, they only obtained tiny denatured protein by cutting gel purification. In contrast, through one step affinity chromatography, we can produce 4 mg/l highly purified GST-HFOXP3 fusion protein, which will further facilitate *in vitro* functional investigation of FOXP3.

DNA Binding Activity of Purified GST-HFOXP3—Correct folding is important for protein to function properly. Only correctly folded proteins have biological activity. To verify the biological activity of the expressed fusion protein, we next assayed the recombinant protein for DNA-binding activity. Electrophoretic mobility shift assays were performed by incubating GST-HFOXP3 with a non-radiolabelled double-stranded DNA probe (TTR-S probe) which corresponds to a consensus FKH domain binding sequence. Consistent with previously published results (*14*, *15*, *34*), the fusion protein bound strongly to the probe and retarded its migration in the gel (Fig. 4A, lane 2). Addition of anti-HFOXP3 McAb in the gel shift reaction results in retardation of the corresponding band (Fig. 4A, lane 4). The same results of electrophoretic mobility shift assays were also obtained by incubating GST-HFOXP3 with a non-radiolabelled double-stranded

Table 1. **Summary of HFOXP3 protein yields from representative 1l of *E. coli* culture.**

	Total protein (mg)[a]	GST-HFOXP3 (mg)	Soluble GST-HFOXP3 (mg)	Inclusion body	Yield (%)[b]
Total lysate	400.0	40.0	28.0	12.0	10
GSH-Sepharose			4.0		1

[a]Estimated from band intensity of GSH-HFOXP3 on SDS–PAGE.
[b]Calculated based on total lysate protein.

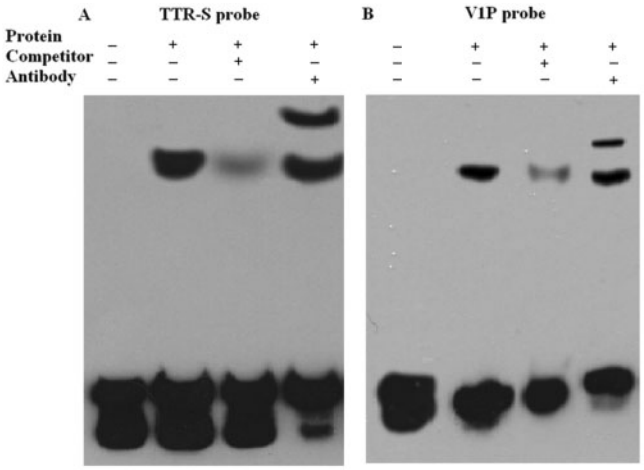

Fig. 4. **Electrophoretic mobility shift analysis of GST-HOXP3 bound to specific DNA sequences.** A dimer of the consensus FKH-binding site in (A) the transthyretin promoter (TTR-S) and (B) the V1 heavy chain gene promoter (V1P) were biotin-labelled and incubated with the recombinant GST-HFOXP3 protein. To evaluate the DNA–protein complexes formed between GST-HOXP3 and the labelled oligonucleotide, a 100-fold molar excess of unlabelled oligonucleotide competitor about (lane 3) was added to the gel shift reaction prior to addition of the probe. Anti-HFOXP3 McAb (lane 4) was added to verify the presence of the DNA–protein complex.

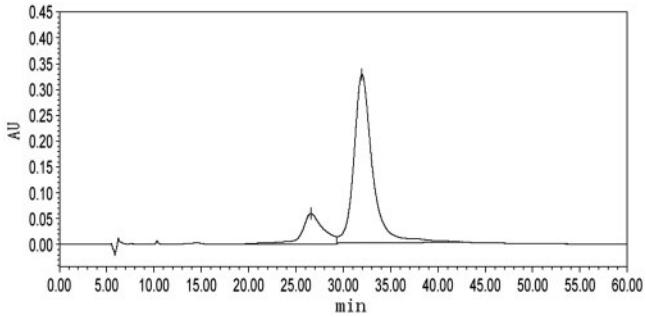

Fig. 5. **Analysis of purified GST-HFOXP3 by reverse phase HPLC on the preparative C4 column.**

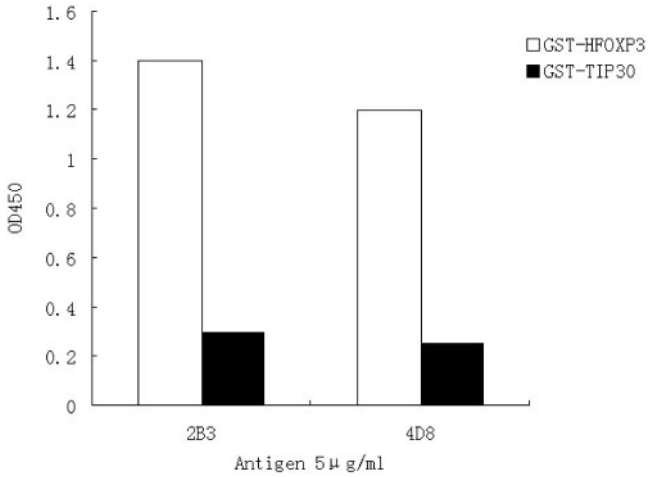

Fig. 6. **ELISA assay for the specific binding activity of the two different McAbs.** GST-HFOXP3 and GST-TIP30 were used in ELISA assay to screen McAb against HFOXP3, and the result suggested that the positive clones reacted with HFOXP3 specifically.

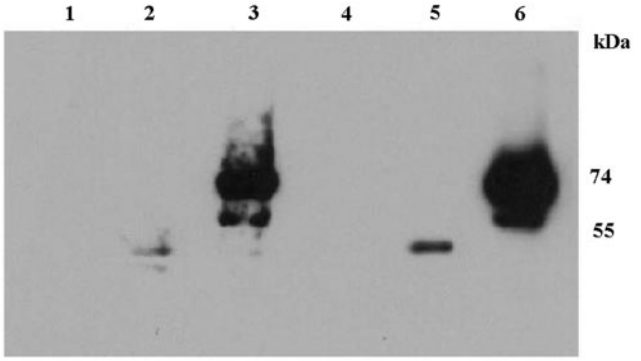

Fig. 7. **Western blotting analysis.** Twenty micrograms of the purified fusion proteins GST-HFOXP3 (lanes 3 and 6), lysate of 5×10^6 PBMC from healthy adult men (lanes 2 and 5) and lymphocytes from mouse spleen (lanes 1 and 4) were separated by 10% SDS–PAGE and electrophoretically transferred to nitrocellulose. The membrane was probed with anti-HFOXP3 McAbs. The lanes 1, 2 and 3 was probed with McAb 2B3 and lanes 4, 5 and 6 with McAb 4D8, respectively.

DNA V1P probe which corresponds to a related sequence reported to bind HFOXP3 with greater efficiency (Fig. 4B). However, any binding and gel shift reaction was not detected when using pure GST protein and adding anti-GST McAb (data not shown). This indicates that HFOXP3 protein but not GST protein is the primary component of the DNA–protein complex in our experiments. Together, these results indicate that most, if not all, purified GST-HFOXP3 proteins were capable of binding specific DNA sequences.

The Yield of Folded HFOXP3 in the Purified GST-HFOXP3—Purified GST-HFOXP3 was separated into two major peaks by reverse-phase HPLC (Fig. 5). The peak with retention time of 31.96 min was found by EMSA assay to be biologically active HFOXP3. But the peak with retention time of 26.59 min was found to have no biological activity in the EMSA assay. The amount of proteins in different peaks was determined by integrating the peak area. So the yield of folded GST-HFOXP3 was estimated to be about 85% of purified GST-HFOXP3 protein.

Generation and Identification of Anti-HFOXP3 McAb—The titre of anti-HFOXP3 antibodies in the serum of the immunized mice was approximately 1:5000 as determined by analysing the half maximum optical density by ELISA. To rule out the clones binding with GST, both fusion protein GST-HFOXP3 and GST-TIP30 were used in ELISA screening. Only that the reader of GST-HFOXP3 is close to that of positive serum while the

reader of GST-TIP30 near to that of medium or negative serum could be regarded as positive clone. After four rounds of subcloning, two stable clones secreting McAb with specificity to HFOXP3 were established, named 2B3 and 4D8, which reacted with GST-HFOXP3, but not with GST-TIP30 in ELISA (Fig. 6). The isotype-determined assay showed that the isotypes of the two clones were both IgG1. The 2B3 and 4D8 cells were injected into the abdomen of BALB/c mice for large-scale antibody production.

The McAb Recognition of Native HFOXP3—Since HFOXP3 protein used in immunization and screening was obtained from prokaryotic cells, its structure may be different than HFOXP3 from eukaryotic cells. It has been reported that HFOXP3 specifically expresses in $CD4^+CD25^+$ T lymphocytes from peripheral blood. To confirm the specific binding of McAb with native HFOXP3, lysate of 5×10^6 PBMC from healthy adult men and purified GST-HFOXP3 was subjected to SDS–PAGE and followed by western blotting with supernatant from the positive clones as primary antibody. After addition of horseradish peroxidase (HRP)-labelled goat anti-mouse IgG, the results from western blotting demonstrated that both the McAb 2B3 and 4D8 could react with HFOXP3 from prokaryotic expression vector and PBMC from healthy adult men. The purified GST-HFOXP3 and native HFOXP3 proteins were about 74 and 55 kDa, respectively. It has been reported that two isoforms of HFOXP3 proteins in Tregs are 55 and 50 kDa and correspond to the products of the native form and the splice isoform of Foxp3 mRNA, respectively (*35*). However, the two antibodies can only detect one

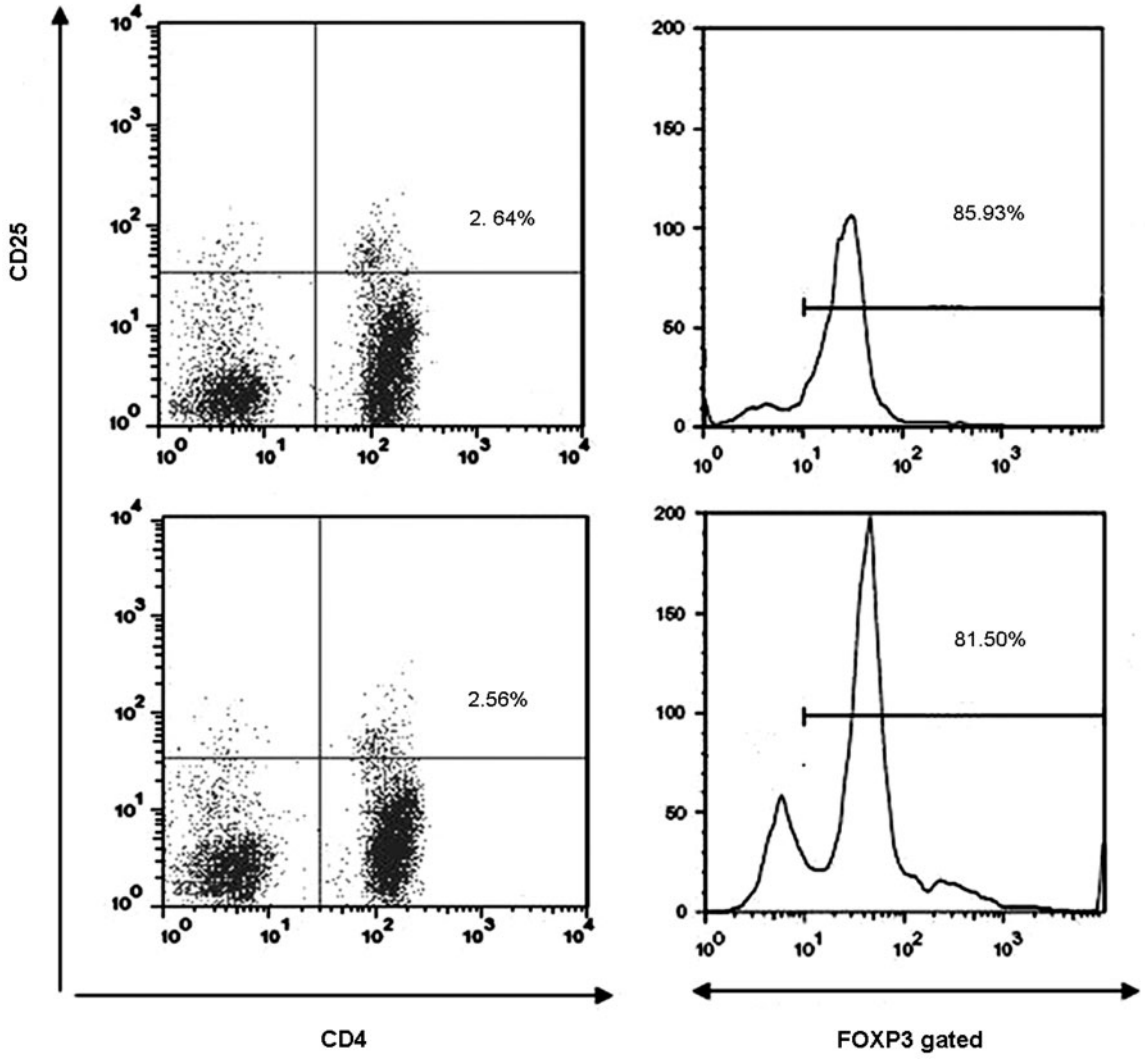

Fig. 8. **Representative flow cytometric profiles of PBMC from healthy adult men for CD4, CD25 and FOXP3**. The PBMC were stained with Cy-Chrome-conjugated anti-human CD4, PE-conjugated anti-human CD25, mouse anti-human FOXP3 McAb and followed by FITC-conjugated goat anti-mouse IgG. (A) Anti-HFOXP3 McAb 4D8. (B) Commercial anti-HFOXP3 McAb. The cells were then washed and analyzed by flow cytometry. The left panel shows the FACS plots and gating of PBMC labelled with CD4 and CD25. The right panel represents FOXP3 protein expression in $CD4^+CD25^+$ T-cell subpopulation. Results are representative of three separate experiments.

protein band about 55 kDa (Fig. 7, lane 2 for 2B3 and lane 5 for 4D8). There is a significant sequence similarity between human FOXP3 and murine FOXP3. As a full-length HFOXP3 protein was used as the antigen it was likely to produce the McAbs which recognize murine FOXP3. To confirm the specificity of McAbs, lysate of 5×10^6 lymphocytes from mouse spleen was also used for western blotting. Results showed that the two antibodies do not have cross-reactivity with mouse FOXP3, which indicates that McAbs have high specificity for HFOXP3 (Fig. 7, lane 1 for 2B3 and lane 4 for 4D8).

Flow Cytometry Analysis of the FOXP3 McAbs—The level of circulating Tregs out of the peripheral $CD4^+$ T-cell pool in healthy humans ranges from 5% to 10% (*11*, *12*). Tregs play a crucial role in the control of T-cell-mediated immune diseases and the amount of Tregs would change in a series of diseases. Therefore, we detected the frequencies of $FOXP3^+$ Tregs in peripheral blood taken from three healthy individuals using the developed McAbs by flow cytometry. A commercial McAb was used as control. As shown in Fig. 8, comparative frequency of $FOXP3^+$ Tregs was detected in gated $CD4^+CD25^+$ T cells by 4D8 clone when compared with commercial anti-HFOXP3 McAb, which were 85.93 and 81.5%, respectively and no detectable staining was observed using 2B3 clone (data not shown). The results here demonstrated that the 4D8 clone was capable of specifically detecting FOXP3 expression in Tregs by flow cytometry, which reached almost the same detection efficiency with commercial available anti-HFOXP3 McAbs.

Immunocytochemistry—To test whether the two clones have more broad applications, the antibodies were used for immunocytochemistry test (*36*). The DNA of HFOXP3 was cloned into eukaryotic expression vector pcDNA3 and transfected into CHO cells using Lipofectamine™ 2000. The resulting CHO cells were stained by anti-HFOXP3 McAbs followed by HRP-conjugated goat-anti-mouse IgG. As shown in Fig. 9, HFOXP3 expression was readily detected in the transfected CHO cells by our and commercial McAbs, which indicated that the two new McAbs could be used in immunocytochemistry. Importantly, it seemed that 4D8 may be better than commercial anti-HFOXP3 McAb for immunocytochemistry because the staining background of 4D8 was much less than that of the commercial McAb.

Purification Using Anti-HFOXP3 McAb Sepharose—To test whether the McAb we prepared could be used to purify and detect HFOXP3, immunoaffinity column coupled with McAb 4D8 was used and the McAb was captured and detected. The functional purified GST-HFOXP3 fusion protein, which was obtained through one-step affinity chromatography described above, was further purified by immunoaffinity chromatography. In a typical round of purification, as shown in the SDS–PAGE profile in Fig. 10, the purified GST-HFOXP3 appeared as a single specific band at position corresponding to molecular masses of 74 kDa. One obvious advantage of the approach described here is that the affinity of the McAb 4D8 is high and relatively small amounts of McAb is needed for further purifying and

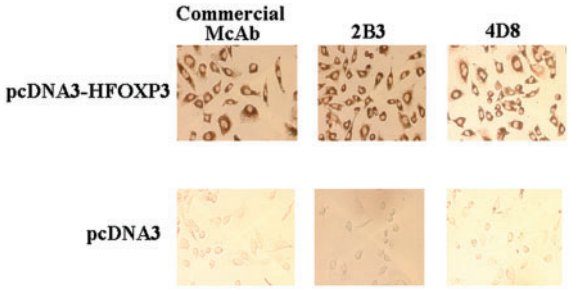

Fig. 9. **Immunocytochemistry of HFOXP3 transfectants**. Anti-HFOXP3 McAbs of 2B3 (middle), 4D8 (right) and commercial McAb (left) were used for detection. The upper row shows the detection of CHO cells transfected with pcDNA3-HFOXP3 plasmid. The bottom row shows the detection of CHO cells which have pcDNA3 transfectants as the control.

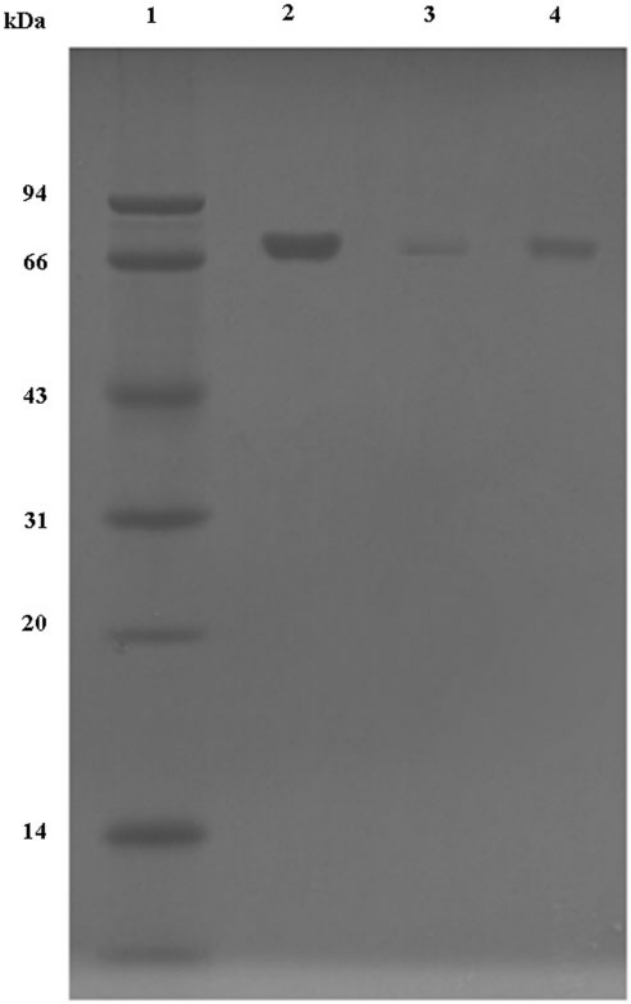

Fig. 10. **Purification using anti-HFOXP3 McAb 4D8 Sepharose**. Lane 1, molecular weight markers; lane 2, GST-HFOXP3 in 0.1 mol/l Tris–HCl buffer (pH 8.3) buffer exchange; lane 3, flow-through fraction from immunoaffinity column; lane 4, elute from immunoaffinity column.

analysing HFOXP3, which had never been reported before.

In conclusion, we have established an effective approach to produce large amounts of functional and intact recombinant HFOXP3 protein and prepared two new anti-HFOXP3 McAbs. The key points in our procedure were: (i) induction of HFOXP3 expression at high cell densities about 1.0–1.2, higher than normal level of OD_{600}~0.6–0.8, (ii) purification of GST-HFOXP3 fusion proteins at low temperature about 4°C, (iii) almost all purified GST-HFOXP3 proteins have the homogeneous conformation and proper biological activity, and (iv) a variety of test procedures to confirm the McAbs in various applications. With this protocol, soluble functional HFOXP3 proteins can be produced at a high level of purity and subsequently used in protein and DNA-binding assays which may provide significant insights into the partner proteins and target genes regulated by HFOXP3. Also, specific anti-HFOXP3 McAb could be used in different detection and diagnosis methods. This, in turn, will provide a valuable tool for defining the roles of Tregs in various diseases and HFOXP3 in development and functional regulation of Tregs.

This work was supported by grants from Ministry of Science & Technology of China (973 & 863 program projects), National Natural Science Foundation of China, Shanghai Commission of Science & Technology, and a special financial support from E-Institute of Shanghai Universities Immunology Division, and Pudong Bureau of Science & Technology. The authors thank Ms Yang Yang and Ms Jing Xu for their technical assistance.

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