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Circumvention of Tolerance for the Nuclear T Cell Protein TCF-1 by Immunization of TCF-1 Knock-Out Mice

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Abstract

Molecular events that underlie the well-defined phenotypic changes of the differentiating thymocyte are poorly understood. A candidate gene to control thymocyte differentiation, *T cell factor-1* (*TCF-1*)* encodes a DNA-binding protein. Its mRNA expression pattern is complex during embryogenesis, yet restricted to lymphocytes postnatally. Expression studies on TCF-1 protein have been hampered by the difficulty to raise antibodies due to extreme evolutionary conservation. *TCF-1* knock-out mice, generated recently in our laboratory, have strongly decreased numbers of thymocytes, but are otherwise normal. We have used these mice to generate anti-TCF-1 antibodies. By immunization with a recombinant fusion protein, we show that *TCF-1* knock-out mice readily yield antiserum titers against human and mouse TCF-1 protein. Wild-type littermates remain unresponsive to TCF-1 while they mount a high-titer antibody response to the fusion protein, Maltose Binding Protein (MBP). Subsequently, TCF-1-specific hybridomas could be prepared from the spleens of immunized knock-out mice. This study illustrates the almost complete tolerance of mice for human TCF-1 and demonstrates that this tolerance is readily broken by gene knock-out. Furthermore, the usefulness of knock-out mice for the generation of monoclonal antibodies against the gene product of interest is underscored.

Introduction

The study of bone-marrow differentiation has provided a unique opportunity for the identification of celltype-specific transcription factors. Recently, studies on the regulation of genes encoding molecules such as T lymphocyte surface receptors have become feasible. T lymphocyte-specific enhancers

* distinct from TCF = ternary complex factor

have been identified in all four T cell receptor-genes as well as in the CD3- δ and the CD3- ϵ genes (reviewed in 1, 2). Subsequent analyses of proteins binding to these enhancers have led to the cloning of at least five novel transcription factors: TCF-1, LEF-1, SOX-4, GATA-3 and Ikaros (reviewed in 3, 4). These factors are candidate genes to control T cell differentiation.

TCF-1 was cloned, based on its affinity for the AACAAAG motif of the human CD3- ϵ enhancer. The primary sequence of TCF-1 suggests a multi-domain structure. The N-terminal half is rich in prolines; the C-terminal half is highly basic and contains the DNA-binding domain. At least four alternative splice forms of human TCF-1 exist, of which TCF-1B represents the most common mRNA species. TCF-1 is a member of a family of genes with homology to High Mobility Group I (HMG) proteins. Importantly, this family also includes LEF-1 and SOX-4. The region of homology within this family consists of an 80-amino acid domain termed the HMG box. The HMG box interacts with DNA in a sequence-specific fashion; its target motif is A/T A/T C A A A G (5, 6). This cognate motif of TCF-1 occurs in most other T cell-enhancers. Recombinant TCF-1 thus binds to motifs previously described in the TCR- α , the TCR- β and the TCR- δ enhancer (7).

As demonstrated in a panel of human and mouse cell lines, the TCF-1 mRNA was expressed uniquely in T cells (8). Surprisingly, a high dynamic and complex expression pattern occurs during mouse embryogenesis. Thus, TCF-1 is diffusely expressed throughout all embryo-derived tissues at day 7.5 postcoitum. At later embryonic stages, TCF-1 is transiently expressed in limbs, neural crest, pharyngeal arches, thymus, lung, kidney, sinus urogenitalis, genital eminence, tooth bud, the adrenal gland, the thoracic vertebrae, the choroid plexus and meninges. TCF-1 mRNA thus occurs in multiple non-lymphoid tissues during development. Nevertheless, Northern blotting of total tissue RNA of adult mice resulted in clear signals in lymphoid tissues only (6). These apparently contradictory observations most likely reflect drastic changes in expression patterns of TCF-1 occurring around birth.

Until now, TCF-1 expression has only been analysed at the mRNA level. The direct visualization of the translated protein (either by gel retardation or by immunological techniques) has remained elusive, as we have been unable to raise antibodies in mouse, rabbit and chicken despite extensive efforts. This probably reflects the conservation of the TCF-1 sequence between species. As TCF-1 is highly expressed in thymus, intrathymic selection mechanisms will impose tolerance for TCF-1 in the immune system, likely through clonal deletion of TCF-1-reactive T cells. To circumvent tolerance-related phenomena, we have now immunized TCF-1 knock-out mice with recombinant TCF-1.

Materials and Methods

Production and purification of the TCF-1 fusion protein

The 900 bp EcoRI insert of phage TCF-1 (5), which encodes the TCF-1A splice form starting at amino acid 102 to the C-terminus was ligated into the EcoRI-digested pIH902 plasmid (New England Biolabs), to yield pIH-TCF. After transformation into bacterial strain DH5 α , bacteria were grown on LB agar plates. Colonies were picked and plasmids were screened for a correct TCF-1 insert by sequence analysis. pIH-TCF was then transformed into TB1 bacteria. Cells were grown in LB containing 100 μ g/ml ampicillin. Upon reaching mid-log phase, the bacteria were induced by IPTG to a final concentration of 0.3 mM to produce the Maltose Binding Protein (MBP)/TCF fusion protein, and allowed to grow for another four hours. Bacteria were harvested by centrifugation (10', 4000 g, 4 °C) and resuspended in ice-cold lysis buffer (10 mM NaPO₄, 15 mM NaCl and 0.05 % Tween 20, pH 7.4). Lysis was achieved by sonification (3 $\times$ 3'), after which the cellular debris was removed by centrifugation (1 h, 15,000 g, 4 °C). The MBP/TCF fusion protein was purified in a single affinity chromatography step according to the manufacturer's instructions. Amylose-coated resin beads (Biolabs) were used to bind the fusion protein, and subsequently eluted with maltose (10 mM). Purification of the fusion protein was tested by SDS-PAGE on a PhastSystem (Pharmacia), using pre-cast 8–25 % gradient gels. Protein bands were visualized by silver staining.

Immunization and generation of monoclonal antibodies

Six week-old C 57/Black 6 mice were immunized by subcutaneous injection of 100 microgram fusion protein in Freund's Complete Adjuvant on day one, followed by a similar injection in Freund's Incomplete Adjuvant at day 10, followed by repeated intraperitoneal injections at weekly intervals of 100 microgram fusion protein in PBS. Blood was collected by supraorbital punctures.

For hybridoma fusion, the spleen was isolated and 1×10^8 splenocytes were fused to an equal number of Ag8 myeloma cells using the standard polyethylene glycol protocol. Selection in hypoxanthine/aminopterin/thymidine was initiated directly after replating the cell suspension into 15 96-well flat-bottom plates. Supernatants were screened 10–14 days after the hybridoma fusion. Positive hybridomas were repeatedly subcloned.

COS cell screening assay

As the substrate for the screening of hybridoma supernatant, COS cells were transiently transfected with a pCDM8 vector encoding human TCF-1B (5) using DEAE-dextran, according to standard procedures. Briefly, COS cells were trypsinized from Petri dishes, washed in PBS and incubated for 60 min at 37 °C in RPMI, containing 0.4 mg/ml DEAE/dextran (Pharmacia) and 1 microgram/ml of the relevant expression plasmid. Cells were then washed once and plated in 96-well flat-bottom culture plates at 10^4 cells/well.

After 48 h, the cells were washed once with PBS, fixed in the plates with 100 % methanol for 20 min at RT and stored under methanol at –20 °C.

For screening, the plates were washed once with PBS, after which 50 microliter of hybridoma supernatant was added. After overnight incubation, the plates were washed once with PBS and incubated for 1 h at RT with horseradish peroxidase-conjugated rabbit-anti-mouse Ig reagent (1:300 in PBS; Dakopatts, Glostrup, Danmark). After two more washes in PBS, a colour reaction was performed with 0.02 % 9-amino-3-ethylcarbazol/0.1 % hydrogenperoxide in 0.1 M sodiumacetate pH 4.8 (9). Individual wells were then examined for staining using an inverted microscope.

ELISA

One microgram protein/ml PBS was coated in a 96-well polyvinylplate for 3 h at 37 °C. After three washes with PBS/0.05 % Tween-20, the plates were blocked with PBS/0.1 % BSA for 1 h at 37 °C. Again three washes before the first antibody (serum) was incubated. Sera or antibodies were diluted in PBS/0.05 % Tween-20/1 % FCS. The serum incubation was 1 h at 37 °C, followed by three washes. The enzyme conjugate, RAMPO (Dakopats), was diluted 1000-fold and incubated for 1 h at 37 °C. Tetra methyl benzidine (TMB) was used as the substrate for the peroxidase reaction. This reaction was stopped after 15 min. at RT by adding equal volume of 1 N H₂SO₄ whereat the optical density was measured at 450 nm.

Results and Discussion

Generation of antiserum titers against TCF-1 in mice

Extensive efforts to produce antibodies against recombinant human TCF-1 protein had previously remained unsuccessful in mice, rabbits and chicken. We therefore decided to utilize Tcf-1 knock-out mice, that have recently been raised in our lab (VERBEEK et al., submitted). We have generated two independent mutations in the germline Tcf-1 gene. One, Tcf^{δVII}, deletes exon VII that encodes part of the HMG box and results in a genuine null-mutation. The second mutation, termed Tcf^{δV}, deletes part of exon V. The resultant mice have moderately impaired numbers of thymocytes at all ages, yet they possess a normal immune system, both in terms of numbers and of functionality of T cells. In these mice, the Tcf-1 gene produces a truncated mRNA (lacking exon V sequences) which in principle still encodes a DNA-binding HMG box protein.

To produce recombinant human TCF-1 antigen for immunization, we cloned the original insert of phage TCF-1 (5) into the plasmid pIH902 (see «Materials and Methods»). The resulting plasmid encoded a chimeric

Figure 1. Graphic representation of the fusion MBP/TCF protein, called MBP-hTCF 1A, and the TCF-1B protein, the most common splice alternative of TCF-1. The first part of the fusion protein is the $\pm$ 430 amino acids MBP protein. The TCF-1 part of the fusion protein starts at amino acid position 102 of TCF-1 (5) and the «HMG»-bar represents the DNA-binding domain of the TCF-1 protein. Only the last 25 amino acids, compare the hatched with the blocked bars at the C terminus, are different between both splice forms.

mouse type	wild type 1	wild type 2	wild type 3	knockout 1	knockout 2	knockout 3
anti MBP titer	$0.6 \cdot 10^6$	$3.0 \cdot 10^6$	$3.0 \cdot 10^6$	$0.6 \cdot 10^6$	$0.6 \cdot 10^6$	$3.0 \cdot 10^6$
anti hTCF-1 titer	2	2	2	$6.4 \cdot 10^3$	$3.2 \cdot 10^3$	$13 \cdot 10^3$
anti mTCF-1 titer	2	2	2	$16 \cdot 10^2$	$8 \cdot 10^2$	$32 \cdot 10^2$

Figure 2. Different antibody titer measured in sera of immunized mice. The MBP titer (hatched bar) was measured by ELISA and there shown to be no difference in titer between wild-type and knock-out mice. Staining of transiently transfected COS cells with hTCF-1 cDNA or mTCF-1 cDNA, yielded the titer for antibodies raised against hTCF-1 (grey bar) or mTCF-1 (blocked bar). Notice the logarithmic scale.

protein consisting of approximately $\frac{2}{3}$ of the human TCF-1A protein fused to the carboxy terminus of Maltose Binding Protein (MBP) (see Fig. 1). The MBP moiety allowed affinity purification and potentially served as an immunogenic carrier. The fusion protein was still capable of binding to DNA in a sequence-specific fashion as determined by gel retardation and subsequent methylation interference footprinting (CASTROP et al., in preparation). This indicated that the recombinant TCF-1 was correctly folded.

We immunized $Tcf^{\delta V}$ homozygous mice and their wild-type littermates with this fusion protein. As demonstrated in Figure 2, anti-MBP antibodies were readily detectable in tail blood samples of all mice after immunization.

Figure 3. This figure demonstrates how easy and clear the COS cell staining can be interpreted. Within one well, one can distinguish between a negative untransfected COS cell (U) and positive nuclear staining of a transfected COS cell (T).

Titers against MBP were determined in an ELISA using a non-fusion MBP preparation. No differences in titer were observed between wild-type and knock-out mice, confirming the healthy immunological status of the $Tcf^{\delta V}$ homozygous mice.

Titers against TCF-1 in the same blood samples were determined by transfecting COS cells with an expression vector containing the TCF-1B cDNA, the most common splice alternative of TCF-1 at the mRNA level. Two days after transfection, COS cells were fixed with methanol and subjected to an immunoperoxidase staining procedure. High titer anti-TCF-1 antibodies were detected in the knock-out mice, whereas (as expected) none of the wild-type mice yielded titers of any significance.

The spleen of one of these mice was subsequently used to rescue anti-TCF-1 antibody-secreting clones. As a screening assay, we again chose immunoperoxidase-staining of methanol-fixed, TCF-1 transfected COS cells. Of several hundred hybridomas screened, four were found to stain nuclei of transfected COS cells. One of these remained positive through subsequent rounds of cloning. This antibody is of IgG2a subclass, indicating that class-switch did occur. Staining of transfected COS cells with supernatant from this hybridoma is demonstrated in Figure 3.

This study illustrates two important points. Firstly, we demonstrate in our model that the absence of the antibody responses against highly conserved molecules like TCF-1 is not due to the inherent absence of

specific TCR- or Ig-V regions from the genome of mice. Rather, responses do not occur because of inducible tolerance mechanisms. This can be concluded, since removal of (part of) the TCF-1 protein from these mice results in readily detectable levels of specific antibodies. Secondly, we show that this approach might be of importance for the generation of antibodies against other highly conserved molecules for which knock-out mice exist and that have resisted classical attempts at raising antisera.

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