New Monoclonal Antibodies to Rat Testicular Antigen, TEC-21

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Background

TES101 is a 38-kDa glycoprotein expressed in mouse spermatocytes and spermatids within the seminiferous tubules but not in any other tissue (Kurita et al., 2001). In mouse testes it is first detectable approximately on day 20 after birth. Its function has not yet been elucidated. However, because the expression is almost parallel to testicular growth, the molecule may have a significant physiological role in sperm formation. This suggestion is supported by recent data indicating that this protein is highly conserved in mammals. We have recently cloned cDNA for a rat homologue, the TEC-21 glycoprotein (BLAST accession number AF347056), which exhibits an 80% identity with TES101 at the amino-acid level. Both TEC-21 and TES101 glycoproteins are found exclusively in testicular tissue and are first expressed at similar developmental stages. Interestingly, TEC-21 is also expressed in rat basophilic leukaemia (RBL) cells, which have been extensively used as a model cell line for analysis of the high-affinity IgE receptor-mediated activation of mast cells and basophils. TEC-21 is a heavily glycosylated glycoprotein bound to the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor. It has apparent molecular masses between 32–36 kDa in rat testes and 36–42 kDa in RBL cells. The TES101/TEC-21-like protein was also identified in human testes (50% of identity with TEC-21 at the amino-acid level; BLAST accession number AAK27310). Thus, monoclonal antibodies (mAbs) against the TEC-21 glycoprotein could become useful reagents in research on the role of this protein in male germ-cell maturation. Because TEC-21, like other GPI-anchored proteins, is associated with lipid rafts in RBL cells, antibodies against TEC-21 would provide a valuable tool for the analysis of the role of lipid rafts in mast cell signalling (Dráber et al., 2001).

Production

BALB/c mice were immunized with lipid rafts obtained after sucrose density gradient ultracentrifugation of RBL cells lysed in 1% Brij 96, as previously described (Surviladze et al., 1998). Gradient fractions corresponding to 15–30% sucrose were pooled and used for immunization and antibody screening. Hybridomas producing TEC-21-specific antibodies were isolated after fusion of spleen cells from immunized mice with myeloma cells SP02. Hybridoma cells were cloned and the antibodies were characterized using standard procedures (Dráber et al., 1980), except that the immunoglobulin isotypes were determined using a mouse mAb isotyping kit (Sigma Chemical Co, St. Luis, MO). Three TEC-21 glycoprotein-specific mAbs were selected. Two of them were of the IgG_{2a} isotype (designated TEC-21 and TEC-22) and one of the IgG_1 isotype (TEC-23).

Specificity

Immunoblotting analyses indicated that all three mAbs reacted with a 36–42 kDa glycoprotein in lysates from RBL cells as well as with 30–36 kDa glycoproteins in testicular tissue from adult rats, but not with any proteins in lysates from numerous other rat tissues and cell lines. The antibodies also reacted with the N-deglycosylated form of the glycoproteins (Fig. 1).

Direct evidence that the mAb recognize the TEC-21 glycoprotein was obtained by flow cytofluorometry analysis of cells transiently transfected with TEC-21 cDNA. As indicated in Fig. 2A, COS cells transfected with the empty vector and stained with TEC-21 mAb showed only background fluorescence. However, after transfection with a vector containing TEC-21 cDNA,

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Abbreviations: BSA – bovine serum albumin, BSS – buffered salt solution, GPI – glycosylphosphatidylinositol, mAb – monoclonal antibody, PBS – phosphate-buffered saline, RBL – rat basophilic leukaemia.

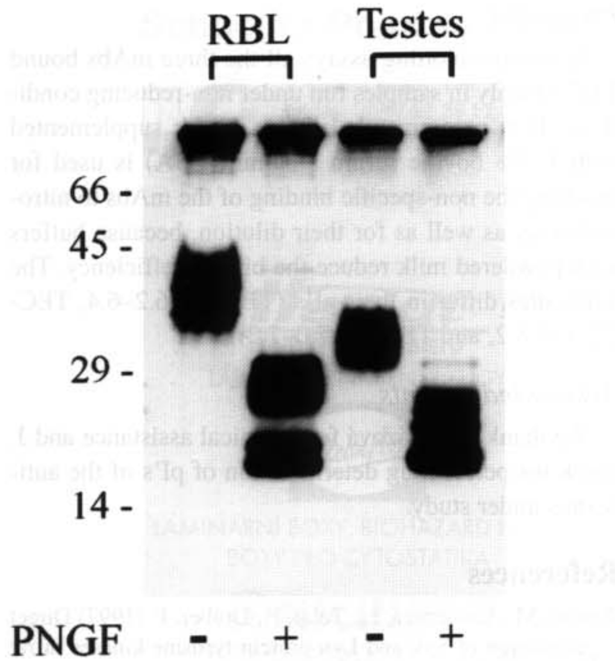

Fig. 1. Immunoblotting analysis of the TEC-21 protein from lysates of RBL and rat testicular cells in glycosylated and N-deglycosylated forms. RBL cells (5 × 10^6) or testicular tissue cells (1.1 mg) were resuspended in phosphate-buffered saline (PBS) supplemented with 0.2% saponin. After 10 min on ice the cells were spun down at 4000 × *g* for 5 min and resuspended in 0.5 ml of ice-cold lysis buffer containing 25 mM Tris (pH 8.0), 140 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin and 1% Nonidet P-40. After 30 min on ice the lysate was spun down at 12000 × *g* for 10 min. The TEC-21 glycoprotein was immunoprecipitated from postnuclear supernatants as described (Tolar et al., 1997) using protein A beads with prebound TEC-21 mAb. Immunoprecipitated material was boiled in 0.5% SDS for 10 min and then treated (+) or untreated (–) for 16 h with peptide N-glycosidase F (New England BioLabs, Beverly, MA; 500 U in 50 μl of a buffer containing 0.05 sodium phosphate, pH 8.0, and 1% Nonidet P-40) to remove N-linked sugar chains. The material was size-separated by sodium dodecylsulphate 12% polyacrylamide gel electrophoresis and analyzed by immunobloting using the standard two-step procedure (Amoui et al., 1997). TEC-21 mAb was used in the form of ascites, diluted 1 : 1000, and goat anti-mouse IgG conjugated with horseradish peroxidase (Transduction Laboratories, Lexington, KY) was diluted 1 : 20000. For blocking the non-specific binding of antibodies to nitrocellulose and for antibody dilutions, a buffer containing 1% BSA and 0.1% Tween 20 was used. Antibody binding was visualized with a chemiluminescence procedure using ECL Western blotting detection reagent (Amersham Pharmacia Biotech, Little Chalfont, England) according to the manufacturer's protocol. Positions of molecular weight standards, in kDa, are shown on the left. Data presented in this figure were obtained using the TEC-21 mAb; similar results were obtained with TEC-22 and also TEC-23 mAb, using a different immunoprecipitation protocol.

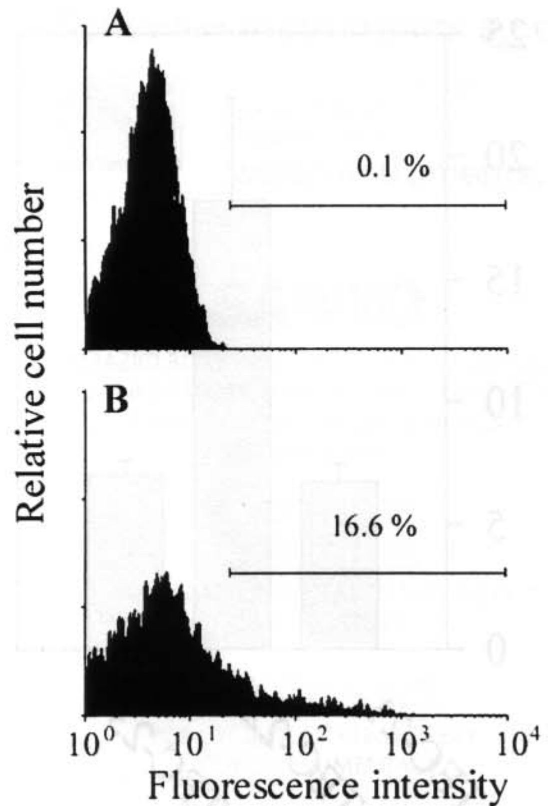

Fig. 2. Flow cytofluorometry analysis of TEC-21 cDNA transfectants. TEC-21 cDNA was cloned from RBL cells (Hálová, Dráberová and Dráber, in preparation) and introduced into an expression vector, pZeo SV2 (Invitrogen, Groningen, Netherlands). COS cells were transiently transfected with the vector using the DEAE dextran method as described (Selden, 1995). After 48 h the cells were harvested and the surface expression of the TEC-21 glycoprotein was evaluated by flow cytofluorometry as described (Tolar et al., 1997). (A) Reactivity of control cells transfected with the empty vector. (B) Reactivity of cells transfected with the vector containing TEC-21 cDNA. Percentage of positive cells is also indicated. Data from a typical experiment, from five performed, are shown.

the number of positive cells was dramatically increased (Fig. 2B). Similar results were obtained with TEC-22 and TEC-23 mAbs.

Although the mouse TES101 and rat TEC-21 glycoproteins exhibit a high level of homology, the TEC-21-specific mAbs did not cross-react with mouse testicular tissue. Protein A-bound TEC-21 or TEC-22 mAbs efficiently precipitated the TEC-21 glycoprotein derived from RBL cells as well as testicular cells lysed in a buffer containing 0.2% saponin followed by a buffer supplemented with 1% Nonidet P-40 (Dráberová et al., 1996).

To determine whether these mAbs recognize different epitopes on the TEC-21 molecule, a direct radioantibody binding assay (Dráber and Stanley, 1984) was employed. Cells were exposed to saturating concentrations of various TEC-21-specific antibodies, and the binding of ^{125}I-labelled TEC-21 was determined.