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From sequence to antibody: Genetic immunisation is suitable to generate antibodies against a rare plant membrane protein, the KAT 1 channel

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Abstract

Monoclonal antibodies against the K⁺ channel KAT1 of *Arabidopsis thaliana*, a low abundance, plant plasma membrane protein, were generated by genetic immunisation to avoid the time and labour consuming purification of native or recombinant proteins and peptides usually necessary for conventional immunisation techniques. The resulting polyclonal and monoclonal antibody sera recognised a single protein band in a microsomal fraction of wild-type *A. thaliana* leaves and in membrane fractions of transgenic yeast cells and tobacco plants expressing the KAT1 protein. Therefore, genetic immunisation is suitable for generating monoclonal antibodies against plant proteins and particularly, against plant membrane proteins of low abundance.

Keywords

Genetic immunisation; K⁺ channel; Monoclonal antibody; Plasma membrane protein

1. Introduction

Antibodies specifically raised against proteins are a well-established tool in biological research enabling the detection of proteins in cells (immunolocalisation) or on membranes after electrophoresis (Western-blot), and also allow their purification via immuno-affinity chromatography. The “classical” way of generating antibodies is labour-intensive and time-consuming. Usually, the whole protein or parts of the protein have to be purified from their natural source or recombinantly produced prior to intradermal injection in rabbits or mice for the generation of antibodies. Alternatively, synthetic peptides representing a specific part of the protein of interest can be injected. All these methods yield a polyclonal antibody serum, whose final properties regarding to its specificity and affinity to the target protein

have to be analysed with the worst case that no injected animal produced antibodies suitable for further biological research. In this case, the entire procedure has to be repeated.

For the generation of antibodies against membrane proteins, including plant plasma membrane proteins, with conventional techniques a low yield of the membrane protein of interest can be expected due to the difficulties in purifying the plasma membrane and due to the low yield of specific membrane proteins, e.g. ion channels [1]. To overcome these difficulties in membrane protein purification, a large hydrophilic part of the protein can be expressed in bacteria and the purified, recombinant peptide can be used for the generation of antibodies. Both procedures were applied to raise antibodies against plant plasma membrane K^+ channels: (1) a purified integral membrane protein from solubilised *Vicia faba* mesophyll cell plasma membranes revealing K^+ transport properties was injected into mice [2] or (2) purified, recombinant N- and C-terminal peptides, respectively were used for immunisation [3-6].

In contrast, the genetic immunisation approach does not require the protein or peptide purification steps. The technology is based on the observation that the inoculation of mice or rabbits with genetic information inserted into a mammalian expression vector leads to in vivo synthesis of the encoded antigens and subsequently to the development of an antigen-specific immune response [7-9]. DNA-based immunisation offers several advantages over conventional immunisation strategies: (i) it is possible to avoid the time consuming and labour-intensive purification of the antigen, (ii) it enables to generate antibodies against proteins where only gene sequences are known, and (iii) the tertiary structure of the protein may serve as antigen [10]. For the generation of high levels of antibodies the plasmid-based antigen expression in a mammalian system requires a strong eukaryotic promoter (cytomegalovirus or the simian virus 40 promoter), introduction of an intron upstream from the translated region and a polyadenylation site that stabilises the mRNA [11,12], and also the route and method of immunisation and the correct presentation of the respective antigen have to be taken into account. DNA delivery can be performed by intramuscular or intradermal saline needle injection or delivered epidermally using a biolistic device [13]. Gene gun immunisation leads to direct transfection by propelling gold beads coated with plasmid DNA into the cytoplasm of the cells, thereby resulting in a much higher transfection efficacy and immunogenicity than needle injection [14]. The generation of antibodies requires generation of T-helper cells via presentation of peptide on MHC II and contact of B-cells with the conformational intact antigen. This can be achieved by targeting the native protein to the cell surface or enable its secretion [15,16]. Both processes require an ER-targeting signal sequence that will induce the translation of the nascent protein chain into the ER, from where it will be transported into the Golgi apparatus where post-translational modifications may take place. A soluble protein that lacks further signal sequences will then be secreted from the cell, while a protein that contains a membrane anchor signal sequence (e.g. a GPI attachment signal), or hydrophobic transmembrane domains may be attached to the cell membrane and transported to the surface. Addition of an ER-targeting signal sequence results in drastically enhanced antibody responses [17] that can be further increased if the protein is secreted [18].

To study the suitability of genetic immunisation for the generation of antibodies against plant proteins, we chose a challenging protein, the *Arabidopsis thaliana* plasma membrane inward rectifying K^+ channel, KAT1 [19], a low-abundance protein with many transmembrane domains.

2. Materials and methods

2.1. Cloning of vector pCMV-TPA/KAT1

Vector pKAT1 containing the cDNA encoding the inward rectifying K⁺ channel KAT1 of *Arabidopsis thaliana* [19] was digested with restriction enzymes *EcoRI/XbaI* resulting in a 520 bp *EcoRI/EcoRI* fragment (165 N-terminal amino acids) and a 1616 bp *EcoRI/XbaI* fragment (512 C-terminal amino acids of KAT1). The 1616 bp and the 520 bp fragments were ligated subsequently into the *EcoRI/XbaI* and the *EcoRI* sites of vector pCMV-MCS3 [20], respectively, resulting in the final immunisation vector pCMV-TPA/KAT1 (Fig. 1A). The vector pCMV-TPA/KAT1 utilises the ER-targeting signal sequence of human tissue plasminogen activator (hTPA). The cloning procedure resulted in the expression of 15 additional amino acids (VDDIACEFLSYVREK) at the N-terminus of the expressed protein.

2.2. Genetic immunisation

Three 6- to 8-weeks-old BALB/c female mice (Charles River, Sulzfeld, Germany) were vaccinated three times at 2 week intervals epidermally using a Helios gene gun (BioRad, Vienna, Austria). Plasmid DNA was precipitated onto gold beads (1.6 µm diameter) by CaCl₂ in the presence of spermidine resulting in 2 µg DNA mg⁻¹ gold. Mice received a total of 2 µg DNA divided between two non-overlapping shots to the shaved abdomen at a helium pressure of 400 psi. Two weeks after the third immunisation sera were checked for the presence of anti-KAT1 IgG by western blot and the mouse with the highest antibody titer was boosted two more times at 7 and 3 days before preparation of hybridoma cells.

Two rabbits were gene gun-immunised three times at two week intervals with six shots (6 µg DNA in total) to the shaved back. Two weeks after the last immunisation blood samples were taken and sera were prepared.

2.3. Fusion of myeloma cells with immune spleen cells

Immune spleen cells were fused with P3-X63-Ag8 myeloma cell line by standard methods. Cells were incubated at 37 °C, 95% relative humidity, 7% CO₂ for 10 days. At the 5th day of incubation 1 ml fresh medium was added. At day 10 supernatants were analysed for anti-KAT1 IgG antibodies by ELISA [20] using a microsomal fraction prepared from *Arabidopsis thaliana* leaves as antigen. Hybridomas from positive wells were cloned by limiting dilution.

2.4. Plant material and membrane fractions

Transgenic tobacco plants were obtained by classical *Agrobacterium tumefaciens*-mediated leaf disc transformation [21] with the pCAM-BIA1302 vector containing a KAT1::GFP fusion construct expressed under the control of two CaMV 35S promoters. Transgenic plants were selected due to hygromycin resistance. Microsomal membranes of *Arabidopsis thaliana* and *Nicotiana tabacum* leaves were prepared by differential centrifugation [22].

The yeast strain PLY246, a $\Delta trk1 \Delta trk2 \Delta tok1$ triple mutant [23] was transformed with a multicopy plasmid (pYES2-KAT1) containing a galactose-inducible promoter. For KAT1 expression the transgenic yeast cells were grown in SGal-medium without uracil plus 50 mM KCl (0.67 w/v yeast nitrogen base without amino acids, 8% w/v galactose, supplemented with 100 µg ml⁻¹ leucine, 20 µg ml⁻¹ tryptophane and 50 mM KCl) at 30 °C and 200 rpm. A yeast membrane fraction was prepared according to Svennelid et al. [24]. Protein was determined with a Lowry Protein Assay (BioRad) with BSA as standard.

2.5. SDS-PAGE and Western blot

Micosomal proteins were separated by SDS-PAGE according to Laemmli [25] and electro-blotted onto nitrocellulose or PVDF membranes (Roth, Karlsruhe, Germany). Membranes were blocked with 0.2% Tween-20 and 5% (w/v) casein hydrolysate or 1% (w/v) BSA in PBS for 3 h at RT. Membranes were incubated with primary antibodies diluted in blocking buffer (1:50 for polyclonal mouse anti-KAT1 and rabbit anti-KAT1 sera; 1:10–1:50 for hybridoma cell supernatants anti-KAT1, and 1:100 for rabbit anti-GFP (Clontech, Heidelberg, Germany)) over night at 4 °C, washed three times with PBS-Tween-20 and incubated with secondary antibodies (anti-mouse IgG or anti-rabbit IgG) coupled to horseradish peroxidase (Promega, Mannheim, Germany) or alkaline phosphatase (Sigma–Aldrich, Vienna, Austria) diluted 1:3000 and 1:8000, respectively, in PBS-Tween-20 for 1 h at RT. After washing in PBS-Tween-20 the secondary antibodies were visualised by chemiluminescence (HRP, ECL-system, Amersham Biosciences, Vienna) or colour development (AP) using standard detection methods. In some blots the average intensity along a 25 pixel wide line overlaid onto the blot image was measured (SigmaScan, Jandel Scientific Corp., USA).

3. Results and discussion

3.1. Construction of DNA vaccine and immunisation of rabbits

The full-length coding sequence of the *Arabidopsis thaliana* inwardly rectifying K⁺ channel KAT1 was placed under the control of the cytomegalovirus (CMV) immediate early promoter-enhancing region (Fig. 1A). The used immunisation plasmid also contains an optimised chimeric intron and the SV40 late polyadenylation sequence of simian virus SV40. The KAT1 cDNA is in frame with an ER-targeting leader sequence (human tissue plasminogen activator, hTPA) directing the KAT1 protein into the secretory pathway. It was shown that the secreted form of an integral protein elicited a much higher antibody titer than the wild type protein [16,26]. As a first test for the quality of the constructed plasmid a polyclonal anti-KAT1 serum was generated in rabbits. The polyclonal serum harvested after the first immunisation recognised only two proteins with molecular masses of 50 and 57 kDa (Fig. 1B, lane 1). Immunodetection with the antiserum harvested after a second immunisation of the same rabbit detected only one protein with an apparent molecular mass of 59 kDa (Fig. 1B, lane 2).

3.2. Analysis of antigen-specific antibody production in mice

For the production of monoclonal anti-KAT1 antibodies, the sera of genetically immunised BALB/c female mice were harvested and checked for the presence of anti-KAT1 IgG by ELISA using an *Arabidopsis thaliana* leaf microsomal fraction as antigen sample (data not shown). Sera of three mice showing a high antibody titer, were subsequently tested in immunoblots against a microsomal fraction of *Arabidopsis thaliana* leaves (Fig. 2), that contain the KAT1 channel protein at a low abundance level [27,28]. All three sera recognised only one protein band at a molecular mass of 65 kDa (Fig. 2, lanes 1–3) whereas no signals could be detected using the pre-immune sera of the three mice (Fig. 2, lanes 4–6).

The mouse with the highest antibody titer (Fig. 2, lane 1) was boosted two more times at 7 and 3 days before preparation of hybridoma cells. Then, spleen cells were fused to myeloma cells and after 10 days of cultivation the supernatants were analysed for anti-KAT1 IgG antibodies by ELISA using a microsomal fraction of *Arabidopsis thaliana* leaves as antigen (data not shown). Hybridoma supernatants of positive cell clones were isolated by limiting dilution and finally six independent anti-KAT1 antibody producing cell lines were obtained. All six monoclonal anti-KAT1 hybridoma supernatants recognised a major protein band with an apparent molecular weight of 60 kDa and minor bands ranging between 55 and 65

kDa in an immunoblot of a membrane fraction obtained from KAT1-expressing yeast strain PLY246-pYES2-KAT1 (Fig. 3A, lanes 1–6). The minor signals might be caused by incomplete processing, folding or post-translational modifications of the expressed KAT1, but not by endogenous ion channels of the yeast cells because the wild type yeast strain tested with the supernatant of hybridoma clone no. 4 showed no signals (Fig. 3A, wt 1). Also, the polyclonal rabbit-anti-KAT1 serum recognised a protein band of ca. 60 kDa in KAT1-expressing yeast cells (Fig. 3A, lane 7), but none in wild type cells (Fig. 3A, wt 2). The specificity of both secondary antibodies (anti-mouse and anti-rabbit IgGs) was verified in lanes 8 and 9 of Fig. 3A, respectively, showing no reaction with yeast membrane proteins. Note, that although only weak signals were detectable, the monoclonal anti-KAT1 sera did not cross-react with proteins outside the range of 55–65 kDa on the blot membrane, thus revealing a high specificity. Even in transgenic yeast expressing KAT1, the amount of the channel protein is expected to be low thus causing weak signals during immunodetection.

In a microsomal fraction of a *N. tabacum* transformant (N.t. 3/5) expressing the KAT1::GFP fusion protein a protein band in the range of about 100 kDa (27 kDa GFP + 78 kDa KAT1) was detected with the polyclonal anti-KAT1 rabbit serum, the hybridoma supernatant no. 4 and an anti-GFP antibody (Fig. 3B). Intensity profiles of the presented lanes are shown to visualise the weak blot signals. The small deviation of around 5 kDa might be caused by the fact that all three microsomal samples were separated on different electrophoresis gels. In the wild type *N. tabacum* plant no specific signals could be detected in this range with all three anti-sera. Although long exposure or incubation times during the chemiluminescence and colorimetric detection were applied, no increase in background was observed despite a “shadow” in the lane wt_rabbit anti-KAT1, which is below the specific signal of 100 kDa.

In transgenic yeast cells and in the native source of KAT1, *A. thaliana* leaves, the “genetically”-raised anti-KAT1 sera mainly recognised a single major protein band with an apparent molecular weight range between 55 and 60 kDa. This apparent molecular mass of the putative K⁺ channel protein band differs from the predicted molecular mass of 78 kDa. Expression of KAT1 in insect cells resulted, however, in a protein band with an apparent molecular weight of 70 kDa when detected with a polyclonal serum obtained by immunisation with a C-terminal recombinant peptide of KAT1 [3]. In addition, a monoclonal antibody recognising a protein responsible for K⁺ uptake in *Vicia faba* mesophyll cells, detected a protein with an apparent molecular weight of 67 kDa [2] also lower than the predicted mass for this K⁺ channel.

In the transgenic tobacco leaves the molecular weight of the detected protein band was much closer to the expected KAT1 molecular weight of 78 kDa (100 kDa–27 kDa GFP). Although, this is not exactly the expected theoretical molecular weight of KAT1, no signals were detectable in wild type organisms that do not express KAT1. Due to the low abundance of KAT1 channels in the prepared microsomal fractions, e.g. KAT1 is almost exclusively expressed in the guard cell plasma membrane of *Arabidopsis* leaves [27], only weak signals were expected. It should be noted that in no experiment cross reactivity with proteins of other molecular weights was observed with the hybridoma supernatants as well as with the polyclonal anti-KAT1 sera.

In conclusion, this first study provides a proof-of-principle for the suitability of the DNA-based immunisation technique for generating specific monoclonal antibodies against low abundance, plant plasma membrane proteins, thus avoiding the labour and time consuming purification of membrane proteins or recombinant peptides. This procedure may be even suitable to raise antibodies for proteins of unknown function for which only the gene sequence is available. The technique of genetic immunisation is continuously improved by

many research groups and may become more and more routine in the production of specific antibodies.

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Fig. 1.

DNA-vaccine plasmid and immunodetection with rabbit sera (A) Plasmid map of pCMV-TPA/KAT1 with the coding sequence of KAT1 under the control of CMV-promoter. The human tissue plasminogen activator (hTPA) guides the protein into the ER of the cell. The vector also contains an optimised chimeric intron sequence, a SV40 late polyadenylation signal sequence, the phage f1 *ori* of replication in *E. coli*, and an ampicillin resistance gene (AmpR). (B) Two protein bands at 50.0 and 57.0 kDa were detected in electrophoretically separated *Arabidopsis thaliana* leaf microsomal fractions after the first immunisation (lane 1). The serum of the second immunisation of the same rabbit recognised only one protein band with a molecular mass of 59 kDa (lane 2). Second antibody: goat anti-rabbit IgG, AP-conjugated. 10 µg protein per slot.

Fig. 2.

Immunodetection in *Arabidopsis thaliana* leaf microsomal fractions with polyclonal mouse anti-KAT1 sera. The polyclonal sera of three different mice immunised with pCMV-hTPA/KAT1 recognised a protein band of 65 kDa in *Arabidopsis thaliana* leaf microsomes whereas sera of naive mice show no antibody binding to *A. thaliana* membrane proteins (lanes 4–6). Second antibody: goat anti-mouse IgG AP-conjugate, 100 µg protein on a preparative gel.

Fig. 3.

Immunodetection in KAT1-expressing, transgenic organisms (A) In the membrane fraction of a KAT1-expressing yeast strain PLY246-pYES2-KAT1, protein bands in the range of 55 and 65 kDa were detected by all 6 different hybridoma cell lines (lanes 1–6) as well as by the polyclonal rabbit anti-KAT1 antiserum (lane 7) with a major protein band at 60 kDa. No protein bands were recognised in yeast wild-type strains when tested with hybridoma supernatant no. 4 (wt1) or the polyclonal anti-KAT1 rabbit serum (wt2). The secondary antibodies alone (lane 8 = anti-mouse IgG-AP, lane 9 = anti-rabbit IgG-AP) gave no signals in membrane fraction of transgenic yeasts. Ten micrograms protein per slot. (B) Microsomal membrane fractions of wild type (wt) and transgenic *Nicotiana tabacum* plants expressing the KAT1::GFP fusion protein (plant no. 3/5) were tested with polyclonal rabbit anti-KAT1, hybridoma cell (H.c.) supernatant no. 4, and a polyclonal rabbit anti-GFP antibody. All three antibodies recognised a protein with a molecular mass of about 100 kDa (arrow), whereas in the microsomal fractions of wild type plants signals in the range of 100 kDa could not be detected. Secondary antibodies: anti-rabbit HRP-conjugate for rabbit anti-KAT1 and rabbit anti-GFP; anti-mouse IgG-AP for H.c. no. 4. One hundred micrograms protein per slot. A corresponding intensity profile of the Western blots is also shown with the wild type in gray and transgenic tobacco in black. The horizontal black line indicates the position of the 100 kDa marker protein.