A Monoclonal Antibody Produced Against Naked2

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Naked2 (NKD2) is a member of the Naked family and negatively regulates canonical Wnt signaling. NKD2 may play a role in embryo development and tumor formation by affecting Wnt signaling. In the present study, we describe the establishment of a monoclonal antibody against NKD2 (anti-NKD2 MAb) through the hybridoma method. The purified anti-NKD2 MAb measured a titer of 2.56×10^5 against NKD2 by indirect ELISA. Western blot analysis, immunoprecipitation, and confocal microscope showed that the anti-NKD2 MAb can specifically combine NKD2 protein in SW480 and LOVO cells. Competitive inhibition assays of Western blot and indirect ELISA showed that the anti-NKD2 MAb can be blocked with NKD2₁₋₂₁₇ protein. The anti-NKD2 MAb would be helpful for further studies on the structure activity relationship, protein detecting, and cell-signaling pathway of NKD2.

Introduction

NAKED2 (NKD2) IS A MEMBER OF THE NAKED FAMILY and negatively regulates canonical Wnt signaling.^(1,2) NKD2, with a molecular mass of 50 kDa, is predominantly localized on the vesicles or plasma membrane.⁽³⁾

Full-length NKD2 and its 1–331 fragment are poorly soluble. NKD2 contains an N-terminal catalytic domain (1–300 residues), a proline-rich domain, and a C-terminal ER targeting domain. The C-terminus of NKD2 is highly disordered. The N-terminal region of NKD2 behaves as an intrinsically unstructured protein but contains most of the NKD2 functional domains, including myristoylation, EF-hand motif, Dishevelled binding region, vesicle recognition, and membrane targeting motif.⁽³⁾

NKD2 binds to multiple proteins and may function as a switch protein through its several functional motifs.⁽³⁾ NKD2 is an inducible antagonist of canonical Wnt signaling and has been showed to act by binding and inactivating Dishevelled, a positive regulator of Wnt signaling.^(4,5) Wnt signaling is involved in virtually every aspect of embryonic development. It controls homeostatic self-renewal in a number of adult tissues,⁽⁶⁾ and its dysregulation contributes to oncogenesis.⁽⁷⁾ This allows the prediction that NKD2 may play a role in embryo development and tumor formation by affecting Wnt signaling. It is also reported that myristoylated NKD2 interacts with the cytoplasmic C-terminal fragment of a Golgi processed form of TGF α , coats TGF α -containing exocytic vesicles, and escorts these vesicles to the basolateral membrane of polarized epithelial cells.^(8,9)

The information above implies that NKD2 may have some potential and important functions, such as affecting cell sig-

naling pathway and cancer development. In order to conduct further studies on the structure activity relationship, protein detection, and cell-signaling pathway of NKD2, our laboratory established a monoclonal antibody against NKD2 (anti-NKD2 MAb).

Materials and Methods

Reagents

RPMI 1640 and fetal bovine serum were purchased from GibcoBRL (Grand Island, NY). Freund's adjuvant (complete and incomplete), HAT medium, goat anti-mouse IgG-labeled peroxidase, and polyethylene glycol 1450 (PEG) were from Sigma (St. Louis, MO). O-phenylenediamine (OPD) and enhanced chemiluminescence (ECL) were purchased from Sangon (Shanghai, China). rProtein A Sepharose column was purchased from GE Healthcare (Stockholm, Sweden).

Materials

The recombinant NKD2 protein (NKD2₁₋₂₁₇) was expressed and purified as described previously.⁽³⁾ SP2/0 cell line, human colorectal carcinoma SW480 cell line and LOVO cell line, and BALB/c mice (8 weeks old, female) were obtained from the Cancer Research Center of Xiamen University (Xiamen, China).

Mice immunization

NKD2₁₋₂₁₇ was used to raise antibodies in four BALB/c mice. Each mouse was first immunized with 40 μ g NKD2₁₋₂₁₇ (in 0.2 mL phosphate buffer saline [PBS]) fully emulsified with 0.2 mL Freund's complete adjuvant by subcutaneous

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injection. After 2 weeks, each mouse was given a booster dose of 20 μ g NKD2₁₋₂₁₇ in incomplete Freund's adjuvant. The booster injection was repeated two times at 2-week intervals and blood was collected for serum after the last immunization. The serum was monitored for antibody titers against NKD2₁₋₂₁₇ by indirect ELISA. Mice with sustained antibody titers above 1×10^4 were selected and intravenously injected with 20 μ g NKD2₁₋₂₁₇ without Freund's adjuvant 3 days before cell fusion.

The indirect ELISA was performed as follows⁽¹⁰⁾: 10 μ g/mL NKD2₁₋₂₁₇ in coating buffer (0.05 M bicarbonate, pH 9.6) was coated in the plates overnight at 4°C. The plates were blocked with 5% skim milk at 37°C for 2 h and washed with PBST (PBS containing 0.05% Tween-20) three times. The supernatants of serum or hybridoma cell culture were incubated in the plates for 1 h at 37°C. After washing, goat anti-mouse IgG-labeled peroxidase was added and incubated for 1 h at 37°C. OPD with 0.03% hydrogen peroxide was added to develop color and the optical density (OD) was measured at 490 nm by a microplate reader (model 680, Bio-Rad, Tokyo, Japan).

Establishment of hybridomas

We adopted a cell fusion technique according to the established protocol of Kohler and Milstein.⁽¹¹⁾ SP2/0 cells were fused with splenocytes from the selected mice at the cell ratio of 1:5 (SP2/0:splenocyte) in 50% PEG. The fusion cells were cultured in HAT medium in 96-well plates. All cell colonies were selected and their supernatants were screened for antibody titers against NKD2₁₋₂₁₇ by indirect ELISA. Three cell colonies with high titer were chosen and cloned by the limiting dilution method,^(11,12) and the colony with the highest titer was transferred into 75 cm² tissue culture flasks with RPMI-1640 medium containing 10% fetal bovine serum. The hybridoma culture supernatants were then collected for isotyping analysis.

Production of MAb

The hybridoma cell line with the highest titer was massively cultured for hybridoma injection. Twenty BALB/c mice were intraperitoneally injected with sterile paraffin oil (0.5 mL per mouse) 7 days before the hybridoma injection. Each mouse was injected with 2×10^6 hybridoma cells. Seven to 10 days later, ascites were collected and centrifuged at 10,000 *g* for 30 min at 4°C.

Purification of MAb

The ascites were further purified by rProtein A Sepharose column according to the manufacturer's protocol to acquire purified MAb. The purity and concentration of the purified anti-NKD2 MAb were analyzed by sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) and Bradford protein assay.

Isotype analysis

We determined the class and subclass of the hybridoma culture supernatants and the purified anti-NKD2 MAb with specific commercially available anti-isotype reagent (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions.

Titer analysis

For the titer analysis, the ascites, the purified anti-NKD2 MAb, and SP2/0 mouse ascites (negative control) were serially diluted and tested by indirect ELISA described above. Data were analyzed and graphed with OriginPro 8.1 software (OriginLab, Northampton, MA).

Western blot and immunoprecipitation analyses

SW480 and LOVO cells (about 1×10^7) were lysed in 1 mL RIPA solution (containing 1% Triton X-100, 1% deoxycholate, and 0.1% SDS; Beyotime, Jiangsu, China) supplemented with a protease inhibitor (PMSF, Sangon, Shanghai, China) for 30 min at 4°C, then centrifuged at 12,000 *g* for 30 min at 4°C to obtain the total cell extracts. The protein concentration in the cell extracts was determined by modified Bradford protein assay.

For the Western blot experiment, 20 μ L total cell extracts were directly loaded for the SDS-PAGE analysis. Then proteins were electrophoretically transferred to PVDF membranes by Mini-PROTEAN (Bio-Rad, Hercules, CA). The membranes were blocked with TBST (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.1% Tween-20) containing 5% skim milk for 1 h, then incubated with the diluted NKD2 MAb (1:1000) or the diluted NKD2 MAb with inhibitor (containing 10 μ g/mL of NKD2₁₋₂₁₇ protein) overnight at 4°C. The membranes were then washed with TBST three times and incubated with goat anti-mouse IgG-labeled peroxidase (diluted 1:3000) for 1 h at 37°C. Proteins of interest were visualized with ECL in Kodak Image Station 4000R (Carestream Health, Rochester, NY).

For the immunoprecipitation experiment, about 1200 μ g protein was incubated for 1 h with 8 μ g of the purified anti-

FIG. 1. SDS-PAGE analysis of the anti-NKD2 MAb. M, protein marker; lane 1, purified anti-NKD2 MAb.

FIG. 2. Titer analysis of anti-NKD2 MAb. Line 1, ascites; line 2, purified anti-NKD2 MAb; line 3, SP2/0 mouse ascites (negative control). The coating concentration of NKD2₁₋₂₁₇ was 10 μ g/mL. Each point represents the OD values with the mean $\pm$ standard deviation from four determinations ($n=4$).

NKD2 MAb, and immune complexes were recovered on rProtein A Sepharose. Immunoprecipitates were washed with lysis buffer four times and recovered by centrifugation, and 20 μ L of immunoprecipitates were used for Western blot analysis.

Indirect ELISA competitive inhibition assay

To verify the specificity of the purified anti-NKD2 MAb, we used NKD2₁₋₂₁₇ protein as inhibitor for competitive inhibition assay. The NKD2₁₋₂₁₇ protein was serially diluted in purified anti-NKD2 MAb (1:2000) and used for indirect ELISA.

Confocal microscopy analysis

SW480 and LOVO cells were respectively cultured on glass cover slides overnight at 37°C and fixed with 4% paraformaldehyde at room temperature (RT) for 30 min. We covered the slides with blocking solution (containing 1% BSA, 5% goat serum, 0.2% NaN₃) at RT for 30 min to block the nonspecific binding. Then slides were washed three times with PBS and incubated with the diluted NKD2 MAb (1:2000) at 37°C for 1 h. After washing, the cells were mounted with fluorescein isothiocyanate (TRITC)-conjugated goat anti-mouse antibody (Boster Bio-Engineering, Wuhan, China) and restained with Hoechst 33258 (Beyotime Biotechnology). The slides with cells were incubated without the anti-NKD2 MAb as control. The fluorescence images were captured at excitation laser of 350 nm and emission laser of 460 nm for Hoechst, and at excitation laser of 550 nm and emission laser of 620 nm for TRITC by using Olympus FV1000 Inverted Confocal Fluorescence Microscope (Olympus, Columbia, SC).

Results

Production of MAb

Ten to 12 days after cell fusion, supernatants of growing colonies were detected for their ability to secrete specific anti-

bodies by indirect ELISA. Three colonies with high titers (designated A3, B2, and B3) were chosen to subclone to 96-well plates. To ensure their stability and specificity, the three hybridoma cells were experimented three times with subcloning and detecting. Then the hybridoma cells with the highest titer (A3-22-11-22) were chosen to produce anti-NKD2 MAb ascites.

Purification of MAb

The ascites were purified by rProtein A Sepharose column to acquire purified anti-NKD2 MAb. The concentration of the purified anti-NKD2 MAb is around 2 mg/mL analyzed by the Bradford protein assay. Then the purified anti-NKD2 MAb was loaded for the SDS-PAGE analysis. Figure 1 shows two bands in lane 1 with molecular masses of ~50 kDa and ~25 kDa, which fit the molecular weight of the heavy chain

FIG. 3. Western blot and immunoprecipitation analyses of the anti-NKD2 MAb. Lane 1, Western blot with SW480 cells; lane 2, Western blot with LOVO cells; lane 3, immunoprecipitates with SW480 cells; lane 4, immunoprecipitates with LOVO cells; lane 5, competitive inhibition of Western blot with SW480 cells; lane 6, competitive inhibition of Western blot with LOVO cells.

FIG. 4. Indirect ELISA competitive inhibition assay of the anti-NKD2 MAb binding NKD2 protein. The coating concentration of NKD2₁₋₂₁₇ was 10 μg/mL and the NKD2₁₋₂₁₇ protein as inhibitor was serially diluted in the purified anti-NKD2 MAb (1:2000). Each point represents the OD values with the mean $\pm$ standard deviation from three determinations ($n=3$).

and light chain of antibodies. The purity of the anti-NKD2 MAb was analyzed by Quantity One software (Bio-Rad, Tokyo, Japan) and the result of the purity is about 90%.

Isotype analysis

The result of the isotype analysis for the hybridoma culture supernatants and the anti-NKD2 MAb by anti-isotype reagent indicated that they are IgG3 isotype.

Titer analysis

The ascites and the purified anti-NKD2 MAb were serially diluted to measure the titers against NKD2₁₋₂₁₇ by indirect ELISA. As shown in Figure 2, the ascites and the purified anti-NKD2 MAb recognized the NKD2₁₋₂₁₇ have a titer of 1.02×10^6 and 2.56×10^5 , respectively.

Western blot and immunoprecipitation analyses

Western blot analysis showed that the positive band of anti-NKD2 MAb reacted with total cell extracts at a molecular weight of ~ 50 kDa (Fig. 3, lane 1, SW480; lane 2, LOVO) and immunoprecipitation also showed a band at a molecular weight of ~ 50 kDa (Fig. 3, lane 3, SW480; lane 4, LOVO). When NKD2₁₋₂₁₇ protein was used as competitive inhibitor, Western blot showed the positive band at a molecular weight of ~ 50 kDa was not detected (Fig. 3, lane 5, SW480; lane 6, LOVO).

Indirect ELISA competitive inhibition assay

When NKD2₁₋₂₁₇ protein was used as competitive inhibitor, indirect ELISA showed the binding ability of the anti-NKD2 MAb was inhibited and the inhibition increased with inhibitor concentration (Fig. 4).

Confocal microscopy analysis

Bright specific binding signals of the anti-NKD2 MAb were detected in SW480 and LOVO cells by confocal microscopy. The

images clearly showed NKD2 proteins localize to the cytoplasm mainly in LOVO cells and membrane mainly in SW480 cells, while the control had no signal by comparison (Fig. 5).

Discussion

In recent years, MAbs serve as a valuable molecular probe to detect compounds by immunological methods. NKD2 has an important potential function in embryo development and tumor formation because it associates with Wnt signaling. But few anti-NKD2 MAbs are available to research its signaling molecules and structure activity relationship.

In our study using NKD2₁₋₂₁₇ protein as immunogen, we successfully developed a stable hybridoma secreting anti-

FIG. 5. Confocal microscopy analysis of NKD2 protein expression in SW480 and LOVO cells with the anti-NKD2 MAb.

NKD2 MAb. Titer analysis of the purified anti-NKD2 MAb showed this MAb had high affinity for NKD2₁₋₂₁₇. Western blot and immunoprecipitation analyses showed that the anti-NKD2 MAb binds a single band at a molecular weight of ~50 kDa and competitive inhibition of Western blot showed the anti-NKD2 MAb could be inhibited by NKD2₁₋₂₁₇ protein, which proved the specificity of the anti-NKD2 MAb against NKD2 protein. Indirect ELISA competitive inhibition assay further supported this fact. Confocal microscopy analysis confirmed that the anti-NKD2 MAb can recognize, combine, and indicate the location of the native NKD2 in cells.

In conclusion, we established an anti-NKD2 MAb that can be developed into a detection reagent to detect NKD2 protein in samples and be also used in future studies on NKD2 in the cell signaling pathway.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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