Generation and Characterization of Monoclonal Antibodies Against Human Chibby Protein

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Chibby (Cby) was originally identified as an antagonist of the Wnt/ β -catenin signaling pathway. It physically interacts with the key co-activator β -catenin and inhibits β -catenin-mediated transcriptional activation. More recently, we demonstrated that Cby protein localizes to the base of motile cilia and is required for ciliogenesis in the respiratory epithelium of mice. To gain further insight into the physiological function of Cby, we developed mouse monoclonal antibodies (MAbs) against human Cby protein and characterized two Cby MAbs, designated 8-2 and 27-11, in depth. Western blot analysis revealed that 8-2 reacts with both human and mouse Cby proteins, whereas 27-11 is specific to human Cby. The epitopes of 8-2 and 27-11 were narrowed down to the middle portion (aa 49–63) and N-terminal region (aa 1–31) of the protein, respectively. We also determined their isotypes and found that 8-2 and 27-11 belong to IgG2a and IgG1 with κ light chains, respectively. Both MAbs can be employed for immunoprecipitation assays. Moreover, 8-2 detects endogenous Cby protein on Western blots, and marks the ciliary base of motile cilia in the murine lung and trachea as shown by immunofluorescence staining. These Cby MAbs therefore hold promise as useful tools for the investigation of Wnt signaling and ciliogenesis.

Introduction

THE WNT/ β -CATENIN SIGNALING PATHWAY plays pivotal roles in embryonic development and adult homeostasis, including cell proliferation, cell fate decisions, and stem cell maintenance.^(1–3) Upon activation of the pathway, the key co-activator β -catenin is stabilized at the protein level in the cytoplasm and translocates into the nucleus where it forms a complex with TCF/LEF transcription factors to stimulate expression of target genes.^(4,5) More recently, dysregulation of Wnt/ β -catenin signaling has been linked to the pathogenesis of a wide range of human diseases, most notably cancer.^(1–3,6)

Chibby (Cby) was originally isolated as a β -catenin interactor through the yeast Ras recruitment system using the C-terminal activation domain of β -catenin as bait.⁽⁷⁾ It is a small protein of 14.5 kDa that is highly conserved throughout evolution from fly to human. Cby represses β -catenin-dependent transcriptional activation via two distinct molecular mechanisms, competing with TCF/LEF factors for binding to β -catenin,⁽⁷⁾ and facilitating nuclear export of β -catenin through interaction with 14-3-3 adaptor proteins.^(6,8) Consistent with Cby being a negative regulator of Wnt/ β -catenin signaling, its loss of function in fly and mice results in ectopic activation of this pathway.^(7,9)

Cby also functions in formation of motile cilia in the nasal and lung epithelium.⁽⁹⁾ Cby-knock-out (KO) mice suffer from chronic upper respiratory tract infection due to poorly dif-

ferentiated ciliated cells characterized by a marked reduction in the number of motile cilia in the respiratory epithelium. In good agreement with these findings, Cby protein localizes to the base of motile cilia, suggesting that Cby is directly involved in motile ciliogenesis. The phenotypes of Cby-KO mice share similarities to clinical features of primary ciliary dyskinesia (PCD).⁽¹⁰⁾

Here, we report the generation of mouse monoclonal antibodies (MAbs) against human Cby (hCby) protein. We narrowed down their epitopes, isotyped, and evaluated their utility for Western blotting, immunoprecipitation, and immunofluorescence staining of mouse tissues. The Cby MAbs should facilitate further study of Cby, Wnt signaling, and ciliogenesis.

Materials and Methods

Plasmids, bacterial expression, and cell line

The Flag-, HA- and Myc-tagged hCby constructs have been described previously.^(7,11) The Flag-tagged mouse Cby (mCby) plasmid was created by subcloning a PCR-amplified mCby cDNA into the EcoRI/XhoI sites of a CS2+Flag vector. To generate the His-CbyN expression plasmid for bacterial production of the antigen, a DNA fragment encoding the N-terminal half of hCby was prepared by PCR and inserted into the NdeI/XhoI sites of pET28c (Novagen, Madison, WI). Similarly, for the GST fusion plasmids with various domains

of hCby (N, C, NN, NC and M), the corresponding DNA fragments were PCR-amplified and subcloned into pGEX4T-1 (GE Healthcare, Piscataway, NJ). All constructs were verified by DNA sequencing. GST fusion proteins were expressed in *E. coli* BL21 cells according to the manufacturer's instructions, and total cell lysates were processed for Western blotting. HEK293T cells were grown in DMEM with 10% FBS and 100 U/mL penicillin-streptomycin and transiently transfected using Expressfect (Denville, Metuchen, NJ).

Development of Cby MABs

The Cby MABs were generated at the Cell Culture/Hybridoma Facility at Stony Brook University. The His-hCbyN (aa 1–63) antigen was expressed in *E. coli* BL21 (DE3), and purified using Ni-NTA His-Bind Resin (Novagen). Immunization, cell fusion, and ELISA screening were performed as described previously.⁽¹²⁾ The isotypes of the Cby MABs were determined using the IsoStrip mouse monoclonal antibody isotyping kit (Roche, Branford, CT).

Western blotting and immunoprecipitation

Western blot and immunoprecipitation analyses were performed as described previously.^(6,8) The primary antibodies used were as follows: rabbit anti-Cby,⁽⁷⁾ mouse anti-Flag M2 (Sigma-Aldrich, St. Louis, MO), mouse anti-GST (Novagen), and mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunohistochemistry

Lung and tracheal tissues were dissected from 2- to 4-month-old mice and fresh-frozen in the Cryo-Gel medium (Instrumedics, Richmond, IL). Frozen sections were post-fixed with paraformaldehyde and processed for double-immunostaining with 8-2 and anti-acetylated α -tubulin (isotype IgG2b; Sigma-Aldrich) antibodies as described previously.⁽⁹⁾ Antigen-antibody complexes were detected with Alexa Fluor 488- and 568-conjugated isotype-specific secondary antibodies (Invitrogen, Carlsbad, CA). The sections were then stained with DAPI (Sigma-Aldrich) and mounted using Fluoromount-G (Southern Biotechnology, Birmingham, AL). Images of representative fields were acquired using an Olympus BX61 microscope equipped with a Cooke Sensicam QE CCD camera.

Results

Establishment of mouse Cby MABs

We prepared the N-terminal half of human Cby (hCby) protein (aa 1–63; CbyN) with a His tag at its N-terminal end and used as an immunizing antigen since this portion of Cby is highly soluble when expressed in *E. coli* and can be produced and purified in large quantities. We screened culture supernatants from a total of 960 hybridoma clones by ELISA and identified 12 positive clones. Subsequently, we tested these ELISA-positive hybridoma supernatants by immunoblotting of total cell lysates from HEK293T cells transfected with full-length hCby. Four of the best antibody-producing hybridomas, 8, 81, 27, and 37, were selected, subcloned by the limiting dilution method to ensure their monoclonality, and one subclone from each hybridoma cell line, 8-2, 81-21, 27-11,

and 37-2, were subjected to further analyses as described below.

Initial characterization and epitope mapping

First, we examined the cross-reactivity of the selected Cby MABs with mouse Cby (mCby) protein using Western blotting. Flag-hCby and Flag-mCby were separately expressed in HEK293T cells, and cell lysates were prepared and resolved on SDS-PAGE. The amount of the cell lysates was adjusted to give a roughly equal intensity of human and mouse Cby bands as indicated by immunoblotting using anti-Flag antibody (Fig. 1, lanes 1, 2). Under these conditions, 8-2 and 81-21 detected mCby with a slightly weaker intensity compared to the human protein (Fig. 1, lanes 3–6). In contrast, 27-11 and 37-2 reacted only with hCby (Fig. 1, lanes 7–10), despite the fact that human and mouse Cby proteins are highly homologous, sharing 82% identity and 95% similarity.

In an attempt to map the epitopes of the Cby MABs, we constructed a series of hCby deletion mutants as GST fusions: N (aa 1–63), C (aa 64–126), NN (aa 1–31), NC (aa 32–63), and M (aa 49–78), as depicted in Figure 2A. These GST-Cby mutants were then expressed in *E. coli*, and total cell lysates were prepared and separated by SDS-PAGE for Western blot analysis. The amount of total cell lysates was adjusted to yield a roughly equal intensity of each GST fusion band on the Western blots with anti-GST antibody (Fig. 2B, lanes 1–3; Fig. 2C, lanes 1–4). As expected, all Cby MABs specifically detected CbyN but not CbyC (Fig. 2B). Furthermore, 8-2 and 81-21 recognized CbyNC and CbyM but not CbyNN (Fig. 2C, lanes 5–12), indicating that their epitopes lie within aa 49–63 (Fig. 2A). On the other hand, 27-11 and 37-2 detected CbyNN but neither CbyNC nor CbyM (Fig. 2C, lanes 13–20), suggesting that their epitopes are located in CbyN (the first 31 amino acid residues of hCby) (Fig. 2A). Visual inspection of this region revealed that the N-terminal domain of hCby is diverged from that of mCby (¹MPFFGNT⁷; non-conserved amino acids are shown in bold) (Fig. 2A). Since 27-11 and 37-2 are specific to hCby protein, we suspect that their epitopes are contained within the most N-terminal region. We also determined their isotypes and found that 8-2 and 81-21 belong to IgG2a(κ), while 27-11 and 37-2 are IgG1(κ).

FIG. 1. Western blot analysis of Flag-tagged hCby and mCby proteins using Cby MABs. Total cell lysates from HEK293T cells transfected with an expression plasmid for Flag-hCby (H) or mCby (M) were separated by SDS-PAGE and subjected to Western blotting using the indicated Cby MABs. Immunoblotting with anti-Flag antibody verified that similar amounts of human and mouse Cby proteins were loaded (lanes 1, 2). The hybridoma culture supernatants were used at a dilution of 1:100.

FIG. 2. Epitope mapping of Cby MAbs. **(A)** Schematic of various hCby deletion constructs used in this study. Black lines represent the regions present in each deletion construct. Amino acid sequences of the epitopes for Cby MAbs, determined by Western blot analyses **(B, C)** are shown on the bottom. Identical and similar residues are highlighted in black and gray, respectively. **(B)** Crude lysates from *E. coli* expressing GST, or GST fusion with the N-terminal half (aa 1–63) or C-terminal half (aa 64–126) of hCby were separated by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. **(C)** Crude lysates from *E. coli* expressing GST, or GST fusion with NN (aa 1–31), NC (aa 32–63), or M (aa 49–78) were resolved by SDS-PAGE, followed by Western blotting using the indicated antibodies. Immunoblotting with anti-GST antibody showed that similar amounts of GST fusion proteins were loaded.

Given the same characteristics described above between 8-1 and 81-21, and between 27-11 and 37-2, it is most likely that 8-2 and 81-21, and 27-11 and 37-2 hybridoma subclones produce the same types of Cby MAbs. Thus, in the following studies, 8-2 and 27-11 were further analyzed in detail.

Analysis of 8-2 and 27-11 Cby MAbs by immunoprecipitation and Western blotting

We next tested whether 8-2 and 27-11 are suitable for immunoprecipitation assays. To this end, Flag-hCby was expressed in HEK293T cells and cell lysates were immunoprecipitated with 8-2, 27-11, negative control mouse IgG, or positive control anti-Flag antibody. Subsequently, the immunoprecipitates were rigorously washed and resolved by SDS-PAGE, followed by immunoblotting with anti-Flag antibody. As shown in Figure 3, both 8-2 and 27-11 were able to bring down Flag-Cby efficiently to a level comparable to anti-Flag antibody.

FIG. 3. Cby MAbs were tested for immunoprecipitation assays. Cell lysates from HEK293T cells expressing Flag-hCby were immunoprecipitated with negative control mouse IgG, 8-2, positive control anti-Flag antibody, or 27-11. The immunoprecipitates were separated by SDS-PAGE and immunoblotted using anti-Flag antibody. IgG L, IgG light chain.

So far, our assays employed only Flag- or GST-tagged Cby proteins. We therefore asked if 8-2 and 27-11 are able to detect untagged Cby as well as other epitope-tagged forms on Western blots. For this, HEK293T cells were transiently transfected with an expression plasmid for untagged, or N-terminally Flag-, HA-, or Myc-tagged hCby, and cell lysates were processed for immunoblotting. Rabbit polyclonal anti-Cby antibody⁽⁷⁾ bound all forms of hCby, demonstrating that these proteins were stably expressed (Fig. 4A). 8-2 also recognized all forms of hCby. In contrast, 27-11 failed to detect untagged hCby yet recognized all the N-terminally epitope-tagged versions. This suggests that N-terminal tagging may cause conformational changes that expose the epitope of 27-11 otherwise buried in its native conformation.

As 8-2 efficiently detects untagged Cby on Western blots, we examined if it recognizes endogenous protein. As we reported previously,^(7,13) rabbit polyclonal anti-Cby antibody recognized endogenous protein (Fig. 4B, lane 1). Similarly, we found that 8-2 is capable of detecting endogenous protein (Fig. 4B, lane 3). Ectopic expression of hCby further augmented the band intensity (Fig. 4B, lanes 2, 4).

Immunofluorescence staining of mouse tissues using 8-2 Cby MAb

Having established that 8-2 is able to detect endogenous protein in addition to both human and mouse Cby proteins on Western blots, we evaluated the application of 8-2 for immunofluorescence staining of mouse tissues. We previously reported that Cby localizes to the base of motile cilia in ciliated

cells in the respiratory epithelium.⁽⁹⁾ Frozen sections of adult mouse lungs and tracheas were co-immunostained with 8-2 (red) and anti-acetylated α -tubulin antibody (green) to reveal motile cilia in ciliated cells. As shown in Figure 5, 8-2 yielded intense signals with low background, and most displayed significant co-localization with acetylated α -tubulin in ciliated cells of both tissues. No Cby staining was observed in lung or tracheal sections from Cby-KO mice (data not shown), suggesting that the 8-2 signal was specific. In addition, similar localization patterns were seen using rabbit polyclonal anti-Cby antibodies (data not shown). We also found that 8-2 can be reliably used for immunofluorescence staining of paraffin sections after antigen retrieval with either a citrate or Tris buffer (data not shown).

Discussion

Cby is an evolutionarily conserved protein with dual cellular functions, acting in Wnt/ β -catenin signaling and cilio-genesis.^(9,14) In the present study, we generated mouse Cby MAbs and evaluated their effectiveness in various applications, including Western blotting, immunoprecipitation, and immunofluorescence staining.

During the course of this study, we selected two best Cby MAbs, 8-2 and 27-11, based on their superior reactivity in ELISA and Western blotting, species specificity, epitope mapping, and isotyping. We found that 27-11 is specific to hCby (Fig. 1), and its epitope lie within the N-terminal region of hCby (aa 1–31; Fig. 2A). Interestingly, although most of the amino acid residues in this region are identical be-

FIG. 4. Western blot analysis of untagged, N-terminally epitope-tagged, and endogenous Cby using Cby MAbs. **(A)** Cell lysates from HEK293T cells expressing each of untagged, Flag-, HA-, and Myc-tagged hCby were separated by SDS-PAGE and immunoblotted with rabbit polyclonal anti-Cby antibody, 8-2, or 27-11. **(B)** Cell lysates from HEK293T cells with or without transfection of hCby were resolved by SDS-PAGE and analyzed by Western blotting using polyclonal anti-Cby antibody or 8-2.

FIG. 5. 8-2 detects Cby protein at the base of motile cilia in the lung and tracheal ciliated epithelia. Lung or tracheal frozen sections were co-immunostained with 8-2 (red) and anti-acetylated α -tubulin antibody (green) that marks cilia; the merged image is shown. No Cby staining was observed in tissue sections from Cby-KO mice (data not shown). Scale bars: lung airway, 5 μ m; trachea, 2 μ m.

tween human and mouse proteins, we noticed that there are three non-conserved amino acid residues in the most N-terminal domain (Fig. 2A). We speculate that 27-11 may eventually recognize these amino acid residues unique to hCby but further deletion studies are required to delineate the precise epitope. We also showed that 27-11 detects N-terminally epitope-tagged hCby but not untagged protein on Western blots, suggesting that N-terminal tagging, irrespective of the type of epitope tags used, might induce conformational changes that expose the epitope. In its native conformation, however, the N-terminal region of Cby seems to be masked perhaps by intramolecular interactions. This raises general concerns regarding the use of tagged fusion proteins as antigens for the production of antibodies. Nonetheless, 27-11 serves as a useful reagent for specifically detecting N-terminally tagged hCby or for preferential detection of exogenously expressed tagged hCby vs. endogenous protein. It is also possible that certain post-translational modifications or protein-protein interactions may facilitate the surface exposure of the 27-11 epitope under normal physiological conditions.

8-2 Cby MAb detects both human and mouse Cby proteins (Fig. 1). It is also effective in detecting endogenous protein (Fig. 4B). We mapped its epitope to the 15-amino acid portion in the middle region of hCby (aa 49–63; Fig. 2A). It is worthy to note that this region is highly conserved among vertebrates,⁽⁷⁾ raising the possibility that 8-2 recognizes Cby homologues in other vertebrate species such as *Xenopus* and zebrafish. In our previous report,⁽⁹⁾ immunofluorescence staining using rabbit polyclonal anti-Cby antibody demonstrated that Cby intensely localizes to the base of motile cilia in the respiratory epithelium. In agreement with this finding, 8-2 reliably labels the ciliary base in ciliated cells of the lung and trachea (Fig. 5). Like other basal body proteins, Cby also localizes to centrosomes.⁽⁹⁾ Therefore, 8-2 Cby MAb should prove to be useful as a marker for centrosomes and basal

bodies. Successful production of Cby MAbs provides a valuable tool for further exploration of Cby function, Wnt signaling, and ciliogenesis.

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Author Disclosure Statement

The authors have no financial conflicts to disclose.

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