Monoclonal Antibodies to DIPA: A Novel Binding Partner of p120-Catenin Isoform 1

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The coiled-coil domain-containing delta-interacting protein A (DIPA) is a transcription factor implicated in developmental regulation. DIPA is the first protein discovered to selectively interact with the p120-catenin (p120) isoform 1, an alternatively spliced form of p120 expressed preferentially in mesenchymal cells. Although a small fraction of p120 can be observed in the nucleus under certain circumstances, the vast majority of it associates with classical cadherins at adherens junctions. We observed for the first time that a discrete fraction of DIPA exists at cell-cell junctions, in addition to its predominantly nuclear localization. Thus, the p120-DIPA interaction may regulate cell signaling and/or transcriptional events, as has been described previously for β -catenin and the LEF/TCF transcription factor family. To facilitate further study of DIPA and to determine the physiological relevance of its interaction with p120, we have generated and characterized a panel of five DIPA-specific monoclonal antibodies (MAbs) that function in immunoblotting, immunoprecipitation, and immunofluorescence assays.

Introduction

A MASTER REGULATOR OF CLASSICAL CADHERIN STABILITY, p120 is important for epithelial homeostasis, development, tumorigenesis, and metastasis. It is the prototypic member of a family that includes other Armadillo repeat-containing proteins ARVCF, δ -catenin, p0071, and plakophilins 1–3.⁽¹⁾ In addition to, but not exclusive from, its regulation of cadherin turnover, p120 modulates activity of the Rho family small GTPases.^(2–5) The human p120 gene (*CTNND1*) encodes multiple spliced isoforms that range in size from about 75–120 kDa. These isoforms comprise combinations of four different ATG start sites (1–4) and three alternatively spliced exons (A–C). Although most cell types contain multiple isoforms, the long p120 isoform 1 and shorter isoform 3 are predominant in mesenchymal and epithelial cells, respectively.⁽⁶⁾ Snail, Slug, and Twist transcription factors are embryonic epithelial-mesenchymal transition (EMT) inducers that cause isoform switching from p120 isoform 3 to isoform 1 via the epithelial splicing regulatory proteins 1 and 2.^(7–9)

During validation of a yeast two-hybrid screen using human p120 isoform 1AB as bait, we detected a p120 isoform 1-specific interaction with delta-interacting protein A (DIPA). DIPA was originally thought to bind the hepatitis delta antigen,⁽¹⁰⁾ but has since been found to have no relationship to hepatitis delta (personal communication, J. Taylor).⁽¹¹⁾ DIPA is a 202-amino acid protein that contains two coiled-coil do-

main and no other identifiable domains. At the protein level, it maintains a high degree of conservation with greater than 97% identity among mammals (Fig. 1). Exogenous DIPA colocalizes with p78/MCRS1/MSP58 to centrosomes, and its over-expression can repress SRF and AP-1 signaling⁽¹²⁾. DIPA can endogenously regulate adipocyte differentiation by inhibiting C/EBP- β and C/EBP- γ .⁽¹³⁾ When induced by p53 activation, DIPA can compete with β -catenin for binding to the TCF4 transcription factor, which effectively down-regulates Wnt target gene expression.⁽¹⁴⁾ Thus, DIPA is mostly found in the nucleus and appears to function primarily in transcriptional regulation.

The DIPA interaction with p120 is interesting in part because p120 is predominantly membrane-associated and functions in cadherin stability and cytoskeletal rearrangement. As an analogy, the well-known association of another cadherin binding partner β -catenin with TCF/LEF family members is critical for canonical Wnt signaling, which drives normal development and colorectal cancer. TCF proteins can bind directly to β -catenin, DIPA, and the transcription factor Kaiso, a BTB/POZ zinc-finger protein first discovered as a p120 binding partner.^(14–16) Notably, the β -catenin destruction complex, which is inhibited by Wnt stimulation and mutated in most colorectal cancer, appears to selectively ubiquitylate and degrade p120 isoform 1.⁽¹⁷⁾ Thus, several lines of evidence suggest that p120 isoform 1, and by association DIPA, is functionally distinct, perhaps analogous in some respects to

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FIG. 1. Alignment of human, canine, mouse, and rat DIPA proteins. Reference sequences are from the NCBI Protein Database, and ClustalW alignment was performed with MacVector software. The non-identical amino acids are shaded, and the coiled-coil 1 (42-90aa) and coiled-coil 2 (117-147aa) domains are boxed.

the cytoplasmic pool of β -catenin involved in canonical Wnt signaling.

To determine the function of DIPA and its relationship to p120, we have generated and characterized the first DIPA-specific monoclonal antibodies (MAbs).

Materials and Methods

Production of antigen

DIPA was cloned in to pBG100 (Center for Structural Biology, Vanderbilt University). The sequence verified clone was transformed into *Rosetta* (DE3) *E. coli* cells. Large-scale expression was carried out in 2 L of autoinduction media⁽¹⁸⁾ at 37°C overnight. Cell pellet was resuspended in 25 mM NaH_2PO_4 , 500 mM NaCl, and 10% glycerol (pH 8.0), with a total volume of 50 mL. Resuspended cells were lysed during two passages under 15 to 20 k psi using an Emulsiflex C3 (Avestin, Ottawa, Canada). Lysate was spun down at 130,000 g for 2 h. The clarified lysate was discarded and the pellet was resuspended in 25 mM NaH_2PO_4 , 500 mM NaCl, and 8 M urea (pH 8.0), and incubated at room temperature (RT) for 1 h. Cells were pelleted and the supernatant was added to cobalt resin (Pierce, Rockford, IL) pre-equilibrated with 25 mM NaH_2PO_4 , 500 mM NaCl, and 8 M urea (pH 8.0) and rotated overnight at RT. The resin was separated with centrifugation, compacted in a disposable column, and washed with 10 column volumes of 25 mM NaH_2PO_4 , 500 mM NaCl, and 6 M urea (pH 8.0). The column was then washed with the same buffer with an additional imidazole gradient from 0 to 250 mM. Twenty-five 2 mL fractions were collected at a flow rate of approximately 1 mL/min. Fractions were then analyzed by dot-blot to ascertain the location of His-tagged protein using an anti-6xHis antibody (Roche, Indianapolis, IN). Fractions containing 6xHis-tagged DIPA were subjected to SDS-PAGE and the cleanest fractions were pooled for immunization, while fractions containing non-specific bands were pooled to screen immune sera.

Immunization and hybridoma preparation

Four A/J mice (Stock #000646, Jackson Laboratory, Bar Harbor, ME) were injected both subdermally and intramuscularly in the thigh with a total of 50 μg of 6xHis-tagged full-length DIPA protein in Freund's complete adjuvant. At the

same time, the mice were bled via the submandibular face vein to obtain a pre-bleed. Sera were extracted by centrifugation using BD Microtainer tubes and evaluated for antigen specific antibody titers using enzyme-linked immunosorbent assay (ELISA) as described below. Four weeks after the initial immunization, the mice were boosted with the same dose of protein but utilizing incomplete adjuvant (also used in subsequent boosts). After a 2-week interval, the mice were again bled, and antibody titers were assessed by ELISA. Additional boosts were given at 8 and 12 weeks after the initial immunization. In each case, antibody sera titers were similarly evaluated 2 weeks after each immunization. A single A/J mouse showing the most selective and concentrated anti-DIPA titers was chosen for a final boost (50 μg) via an intraperitoneal injection without adjuvant. Four days after this final boost, spleen cells were harvested and electrofused⁽¹⁹⁾ with Sp/20 (courtesy of Dr. William Sutherland, University of Virginia) or NS1 (courtesy of Dr. Robert Jeffery Hogan, University of Georgia) murine myeloma cells. The products of the fusion were plated into methylcellulose-based semi-solid media (ClonaCell, Stemcell Technologies, Vancouver, Canada) containing the selective reagents hypoxanthine, aminopterin, and thymidine (HAT). After approximately 10 days, colonies of interest were picked and distributed individually into 96-well plates based on *in situ* interaction with fluorescently labeled mouse IgG-Fc 488 DyLight (Jackson Laboratory, catalog #515-485-062) utilizing the ClonePix instrument (Genetix, Sunnyvale, CA). Individual clones were expanded in liquid media containing serum, hypoxanthine, and thymidine (Medium E, Stemcell Technologies) and maintained at a density of $5 \times 10^5 - 1 \times 10^6$ cells/mL for generating antibody-rich supernatants. Supernatants from hybridomas were assayed for antigen-specific antibodies by solid-phase ELISA using full-length DIPA in sodium dodecyl sulfate (SDS) buffer as bait. Positively scoring hybridomas were rescreened for performance by Western blot analysis, immunofluorescence, and immunoprecipitation. The most promising anti-DIPA clones were selected, extensively subcloned to ensure monoclonality, and cryopreserved.

ELISA procedure

The following solutions were prepared as follows from chemicals obtained from commercial sources: carbonate-bicarbonate

coating buffer (pH 9.6) was prepared from Na_2CO_3 (1.59 g/L), NaHCO_3 (2.39 g/L), and thimerosal (0.10 g/L); PBS-Tween (pH 7.4) was prepared from NaCl (8.00 g/L), KH_2PO_4 (0.20 g/L), Na_2HPO_4 (1.15 g/L), KCl (0.20 g/L), Tween-20 (1.00 mL/L), and thimerosal (0.10 g/L); BSA layered (5.0 g) on PBS-Tween (500 mL); 1 mM ABTS solution in 70 mM citrate-phosphate buffer (pH 4.2) was prepared from citric acid (5.64 g/L), Na_2HPO_4 (5.84 g/L), and AzBTS- $(\text{NH}_4)_2$ (0.548 g). For ELISA experiments, ELISA quality plates (Immulon 2HB flat bottom microtiter plates 96-well or 4HB flat bottom 384-well plates) (Nunc, Rochester, NY) were coated with 10 mL per plate of a 5 $\mu\text{g}/\text{mL}$ solution of DIPA antigen in carbonate-bicarbonate coating buffer and incubated at 4°C overnight. Plates were washed three times with 100 μL of PBS-Tween with a Bio-Tek ELx 405 automatic microplate washer (Winooski, VT) and incubated with 100 μL of PBS-Tween for 30 min at 37°C, after which the PBS contents were discarded. Aliquots of murine sera dilutions or hybridoma supernatants (depending on the stage of the antibody development process) were incubated in the coated wells in a final volume of 100 μL in PBS for 60 min at 37°C. The plates were then washed three times with PBS-Tween, followed by the addition of aliquots of diluted horseradish peroxidase (HRP)-conjugated affinity-pure goat anti-mouse IgG Fc region-specific secondary antibody diluted in PBS-Tween/BSA at 1:5000 incubated at 37°C for 60 min. After washing the plates three times, ABTS solution was prepared (1.8 mL of H_2O_2 was added per 1 mL of ABTS) and immediately added to each well in 100 μL aliquots. To determine peroxidase activity, absorbance at 414 nm was measured after 15 and 30 min from each well using a Bio-Tek Powerwave HT 340 plate reader with Gen5 software (Winooski, VT). Isotyping was done via ELISA per manufacturer's protocol (mouse isotyping kit, catalog #37503, Pierce).

Cells, tissue culture, and antibodies

All cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2% L-glutamine, penicillin (100 U/mL), and streptomycin (100 mg/mL; Gibco BRL, Gaithersburg, MD). The cell lines used to assess immunoblotting and species cross-reactivity were A431 epidermoid carcinoma cells (human), HCA7 colon carcinoma cells (human), HepG2 hepatocarcinoma cells (human), HT29 colon carcinoma cells (human), IEC6 intestinal epithelial cells (rat), MDCK kidney epithelial cells (canine), and NIH3T3 fibroblasts (mouse). Anti-Flag MAb (M2 cat. #F1804) was purchased from Sigma-Aldrich (St. Louis, MO). Anti-MBP (12B12), anti-Tubulin (DM1 α), and KT3 MAbs⁽²⁰⁾ were obtained from the Vanderbilt Antibody and Protein Resource (Nashville, TN).

Immunoblotting, immunoprecipitation, and immunofluorescence

Procedures for immunoblot analysis, immunoprecipitation, and immunofluorescence have been described previously in detail.⁽¹⁶⁾ Briefly, cells were lysed in a buffer containing 0.5% Nonidet P-40, 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium vanadate, 0.1 trypsin inhibitor unit of aprotinin, and 5 mg of leupeptin per mL. Whole cell lysates were separated by sodium dodecyl

sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Blots were briefly blocked at 4°C with 5% non-fat dried milk in TBS (pH 7.4) and incubated overnight at 4°C with hybridoma supernatant or primary antibody (0.2–2.0 mg/mL) in 3% milk/TBS. The membranes were then washed five times with TBS before incubation with the secondary anti-mouse 680 and/or anti-rabbit 800 antibody (Licor, Lincoln, NE) in Odyssey blocking buffer/TBS for 30 min at RT. Blots were finally washed three times with TBS plus 0.1% Tween-20 and three times with TBS and then processed with the Odyssey immunodetection system (Licor, Lincoln, NE) according to the manufacturer's protocol. For immunoprecipitation, hybridoma supernatant or primary antibody was incubated with magnetic Dynabeads pre-conjugated to Protein G (Vanderbilt Antibody and Protein Resource, Nashville, TN) at 4°C for 2 h. Dynabeads were then washed three times in NP-40 buffer and combined with whole cell lysates that were extracted as described above. Dynabeads and lysate were incubated at 4°C for 2 h before being washed three times with NP-40 buffer and solubilized in Laemmli sample buffer (LSB).⁽¹⁶⁾ For immunofluorescent labeling, cells were plated and cultured for 2 days on glass coverslips before fixing with 3% paraformaldehyde (PFA) and permeabilization with 0.2% Triton X-100 in PBS (pH 7.4). Primary antibody and hybridoma supernatant incubations were performed at RT for 30 min in 3% milk/PBS at 0.5–1.0 mg/mL. After washing three times with PBS, the coverslips were incubated with Alexa Fluor donkey-anti-mouse 594 and/or Alexa Fluor goat-anti-rabbit 488 (Life Technologies) secondary antibodies in 3% milk/PBS at 1:800 dilution for 30 min at RT. The coverslips were finally washed three times with PBS, mounted on glass slides with Prolong Gold (Life Technologies), and visualized using a Zeiss Axioplan 2 microscope (Zeiss, Thornwood, NY).

DIPA polyclonal antibody production and purification

The full-length 6xHis-tagged DIPA protein described above was used to immunize two New Zealand white rabbits (Covance, Princeton, NJ). Sera was collected from both rabbits and tested by Western blot and immunofluorescence. Polyclonal antibodies (PAbs) were subsequently purified from anti-sera by affinity chromatography on Protein-G sepharose columns (AKTA Xpress, GE Life Sciences, Piscataway, NJ).

DNA constructs and recombinant protein production

Full-length human DIPA (encoded by the *CCDC85B* gene) was purchased from the Dana Farber/Harvard Cancer Center DNA Resource Core (Cambridge, MA) in the form of pENTR223-CCDC85B-fusion (clone #HsCD00288507). To create 3xFlag-DIPA, *CCDC85B* was PCR-cloned into the LZRS retroviral vector upstream of an IRES-Neomycin resistance cassette. Stable DIPA knockdown was performed with the pLentiLox 5.0 vector containing a canine DIPA-specific shRNA sequence: 5'-GGGAGAACCCTGGCGCTTAA-3'. Viral production and cell transduction protocols have been described elsewhere.^(21,22)

Maltose-binding protein (MBP)-tagged DIPA protein and fragments were created by PCR cloning the *CCDC85B* gene into the pMal-c2 vector (New England BioLabs, Ipswich, MA).

Results

Initial characterization

Four mice were immunized with full-length 6xHis-tagged DIPA protein solubilized in 6 M urea. One mouse was picked based on anti-sera immunofluorescence and Western blot analysis. Hybridoma generation yielded 423 ELISA-positive clones, but only 42 clones were effective in Western blot and immunofluorescence. Further screening that included immunoprecipitation yielded five MAbs that were chosen for detailed characterization: 2G7, 3E3, 5E11, 8E11, and 15F11. The results of these studies are presented in Figures 2–6, and their characteristics are summarized in Table 1.

Immunoprecipitation and Western blot analysis

To test the ability of these MAbs to detect denatured DIPA in an immunoblot assay, over-expressed 3xFlag-tagged DIPA was immunoprecipitated from transduced MDCK cells and subjected to Western blot analysis (Fig. 2A). The membrane was cut into strips and probed by each of the MAbs. The strip probed with an anti-Flag MAb was used as a positive control, and a DIPA rabbit polyclonal antibody (PAb) is shown for comparison. With the exception of MAb 2G7, all MAbs rec-

TABLE 1. ANTI-DIPA MAB CHARACTERISTICS

Clone	Epitope	IP	WB	IF	Isotype
2G7	CC2	–	–	+	IgG1
3E3	C-term	++	+++	+++	IgG2b
5G11	C-term	+	–	+	IgG1/IgG2a
8E11	C-term	+	–	+	IgG2b
15F11	CC1	+	+	–	IgG1

Epitopes refer to recombinant DIPA fragments described in Figure 6B.

IP, immunoprecipitation; WB, Western blotting; IF, immunofluorescence; (–), no detection; (+), minimal detection; (++) , good sensitivity or specificity; (+++) , good sensitivity and specificity.

ognized two bands: a monomeric 3xFlag-tagged DIPA of about 30 kDa and a higher molecular-weight species of about 75 kDa, which we believe may be a highly insoluble oligomer.

To identify the MAbs that recognize and immunoprecipitate 3xFlag-DIPA, we bound each MAb or an irrelevant mouse IgG MAb (KT3) to magnetic Dynabeads and subsequently incubated each with cleared lysate from MDCK cells expressing 3xFlag-DIPA. These lysates were then separated by SDS-PAGE, transferred to nitrocellulose, and probed by Western

FIG. 2. Reciprocal immunoprecipitations of exogenous DIPA. **(A)** Anti-Flag MAb was used to immunoprecipitate 3xFlag-DIPA from NP-40-detergent cell lysates of transduced MDCK cells, and immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose. Dashed lines represent where individual strips of the nitrocellulose membrane were realigned after being probed separately. Anti-Flag and DIPA PAb were used as controls. The DIPA PAb was probed on the same strip as MAb 15F11 but detected on a separate channel using the Odyssey imaging system. **(B)** A portion of the same MDCK lysate **(A)** was immunoprecipitated with each MAb, anti-Flag, or an irrelevant IgG control. Precipitates were subjected to Western blotting as done above **(A)**, but probed with anti-Flag MAb. MW, molecular weight marker.

blotting with the anti-Flag MAb (Fig. 2B). Again, MAb 3E3 detected the DIPA oligomer, whereas the other MAbs could not. 2G7 was unable to immunoprecipitate 3xFlag-DIPA.

To determine the ability of these MAbs and the DIPA PAb to detect endogenous DIPA, we immunoblotted equal amounts of lysate from wild-type cell lines of rat, mouse, canine, or human origin (Fig. 3). Cell lysate from MDCK cells over-expressing 3xFlag-tagged DIPA was used as a positive control. Lysate from MDCK cells with stably expressed shRNA against canine DIPA was used to test for specificity and to determine the migration of endogenous DIPA. Endogenous mo-

nomeric DIPA (approximately 25 kDa) migrates faster through the gel presumably because it lacks the 3xFlag epitope and amino acid linker in the exogenous DIPA. MAb 3E3 does not react with a number of faint "background" bands recognized by the DIPA PAb (Fig. 3B, F). MAb 3E3 was not able to detect DIPA from IEC6 or NIH3T3 cells, suggesting that either these cells express low levels of DIPA or MAb 3E3 may not cross-react with rodent DIPA. MAb 3E3 detected a faster migrating band that may represent a post-translationally modified DIPA, as previously reported.⁽¹²⁾ Notably the 75 kDa DIPA oligomer was not detectable in wild-type lysates. Also we noted that HCA7

FIG. 3. Detection of endogenous DIPA by MAbs. Cell lysates were isolated from IEC6 (rat), NIH3T3 (mouse), HCA7 (human), HT29 (human), A431 (human), HepG2 (human), and three transduced MDCK (canine) cell lines with RIPA detergent. Lysates were separated by SDS-PAGE (60 μ g per lane) and transferred to nitrocellulose. (A–E) Membranes were probed with MAbs 2G7, 3E3, 5G11, 8E11, and 15F11, respectively. (F) The same membrane used in E that was probed with DIPA PAb and anti-tubulin but detected on a separate channel using the Odyssey imaging system. 3x, lysate with over-expressing 3xFlag-DIPA; EV, lysate with empty vector; sh, lysate with stable expression of shRNA against canine DIPA.

FIG. 3. (Continued).

human colorectal cancer cells express a relatively large amount of DIPA (Fig. 3B, E, F).

Immunofluorescence

To test the efficacy of the DIPA MAbs in immunofluorescence staining, we incubated fixed and permeabilized cells with the five different MAb hybridoma supernatants (Fig. 4). The MAbs showed predominantly nuclear and cytoplasmic staining in most cell lines, which is consistent with previous reports.^(12–14) Surprisingly some antibodies recognized DIPA at cell-cell junctions, which is unreported in the literature. MAbs 2G7 and 3E3 produced junctional staining patterns in MDCK cells. MAbs 2G7 and 5G11 staining appeared at junctions in A431 cells, but 3E3 did not. Interestingly, 3E3 did not detect a robust nuclear pattern in NIH3T3 cells, but rather

a low-intensity diffuse cytoplasmic staining with occasional nuclear accumulations. 15F11 produced low-intensity staining in all tested cell lines and had little nuclear staining.

We further characterized the MAb 3E3 by immunofluorescence because of its junctional staining pattern in MDCK cells, which is similar to that of p120.⁽⁵⁾ We co-stained control and DIPA-knockdown MDCK cells with MAb 3E3 and the DIPA PAb (Fig. 5). As expected, the polyclonal has high sensitivity but poor specificity, evident by the persistent nuclear and Golgi-like staining in the DIPA-knockdown cells. However, the DIPA PAb junctional staining does not appear in the MDCK shDIPA cells. The MAb 3E3 staining in all cell compartments almost completely disappears in the DIPA-knockdown cells (Fig. 5). Thus, we believe that both the nuclear and the cell-cell junctional staining represent endogenous DIPA.

FIG. 4. Performance of anti-DIPA MAb 2G7 in immunofluorescence assays. DIPA MAb 2G7 was incubated with PFA-fixed and TritonX-100-permeabilized cells as labeled. Goat-anti-mouse Alexa Fluor 594 secondary antibodies were used to detect DIPA MAb 2G7 in all panels. No primary antibodies were used in the bottom panels (2nd Ab). Insets show magnifications to better visualize DIPA junctional staining (arrows).

Epitope mapping

To determine the epitopes to which the DIPA MAb 2G7 bind, MBP-tagged full-length recombinant DIPA and five frag-

FIG. 5. Comparison of MAb 3E3 and DIPA PAb by immunofluorescence. MDCK cells stably expressing either non-silencing shRNA control (top) or canine DIPA-directed shRNA (bottom) were fixed, permeabilized, and probed with either DIPA PAb or the 3E3 MAb.

ments (Fig. 6A) were produced using *Rosetta E. coli*. The tagged proteins were purified from bacterial lysates with maltose resin extraction, separated by SDS-PAGE, and detected by Western blotting using each of the five MAb 2G7 and an anti-MBP MAb (Fig. 6B). Because the MBP tag is 42 kDa, all proteins migrated slower than endogenous DIPA. All five MAb 2G7 were able to detect the full-length DIPA. MAb 2G7 only detected the Δ C-term, CC2, and Δ CC1 fragments, and therefore its epitope lies between amino acids 118 and 149. MAb 3E3, 5G11, and 8E11 only detected Δ CC1 and C-term fragments, so they bind to DIPA between amino acids 142 and 195. MAb 15F11 detected the CC1 and Δ C-term fragments; therefore its epitope must be within the first 118 amino acids. Some amount of protein degradation is evident in all lanes. The higher molecular weight DIPA oligomer is appreciable in lanes 1, 4, and 5 of the anti-MBP blot, which suggests that this species requires the CC2 domain for oligomerization. Thus, these five DIPA MAb 2G7 recognize a diversity of epitopes that span the whole protein.

Discussion

To better understand the physiologic function of DIPA and its relationship to p120 isoform 1, we have generated a novel panel of MAb 2G7 against human full-length DIPA. Of the five

FIG. 6. Epitope mapping of the DIPA MAbs. (A) Schematic of full-length DIPA and five overlapping fragments. The coiled-coil regions are labeled "CC1" or "CC2," and the terminal amino acid residues are indicated next to each fragment. (B) Purified lysates from *Rosetta* BL-21 cells were separated by SDS-PAGE, transferred to nitrocellulose, and probed with each of the DIPA MAbs or anti-MBP as control. Lanes 1–6 were loaded with lysates containing MBP-tagged full-length, CC1, ΔC-term, CC2, ΔCC2, and C-term DIPA, respectively.

MAbs characterized here, MAb 3E3 appears to be the most sensitive and specific by Western blot analysis and immunoprecipitation. Although the data shown were generated with antibody supernatants, we have validated the results with purified MAb 3E3 and determined an optimal working concentration of 0.25 $\mu\text{g}/\text{mL}$ for immunofluorescence and 1.0 $\mu\text{g}/\text{mL}$ for Western blotting. Interestingly, 3E3 was the only MAb that could immunoprecipitate the DIPA oligomer from MDCK cells over-expressing 3xFlag-DIPA (Fig. 2B). Whether this higher molecular weight species exists endogenously is unknown, but it clearly forms when DIPA is over-expressed because it is recognized by three distinct antibodies (i.e., anti-Flag, DIPA PAb, and anti-MBP; Figs. 2, 3F, and 6A). Once formed, the DIPA oligomer is highly insoluble, as it is unaffected by boiling in LSB containing β -mercaptoethanol and isolation by SDS-PAGE.

MAb 3E3 performed well in nearly all assays but does not appear to detect endogenous DIPA from the IEC6 (rat) or NIH3T3 (mouse) cell lines by Western blot. The MAb 3E3 epitope lies within the C-term region of DIPA (amino acids 142–195), and V158 is the only non-identical residue in rodent DIPA compared to human and canine. It is possible that the 3E3 MAb requires a glycine at position 158 for binding. On the other hand, the abundance of DIPA in IEC6 and NIH3T3 cells may be below the threshold for immunoblot detection, because MAb 3E3 does detect an immunofluorescence signal in NIH3T3 cells (Fig. 4). Further studies with shRNA and a DIPA point mutant will be required to answer this question. Experimentally, it would be an advantage if MAb 3E3 recognizes only human and canine DIPA while the PAb detects all mammalian DIPA.

The faster migrating band detected by MAb 3E3 (Fig. 3B) is likely to reflect phosphorylation of DIPA at T12. Exogenous

DIPA with a T12A point mutant has been detected as a single band by Western blot.⁽¹²⁾ The T12 residue is predicted to be a casein kinase I and II substrate by NetPhos 2.0 (University of Denmark) and PhosphoMotif Finder (Human Protein Reference Database, Johns Hopkins University).

MAbs 2G7, 3E3, and 5G11 behaved similarly in immunofluorescence applications. In addition to the more prominent nuclear staining, all three detected DIPA at cell-cell junctions. Although DIPA was not readily detected at junctions in all cell types, the result was clear in A431 and MDCK cells. As noted, shRNA controls revealed MAb 3E3 to be highly specific when compared to the DIPA PAb (Fig. 5). The differential junctional staining among MAbs 2G7, 3E3, and 15F11 in A431 and MDCK cells may reflect cell compartment-specific modifications or binding partners that differentially obscure epitopes.

In summary, we have generated five DIPA MAbs and characterized their performance in commonly used antibody-based assays. They comprise diverse isotypes and interact with at least three distinct epitopes. Of these, MAb 3E3 appears to be the best overall with respect to both specificity and performance in a variety of methods.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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