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Choosing the right antibody for Resistin-Like Molecule (RELM/FIZZ) family members

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Abstract

The family of resistin-like molecules (RELM), also known as found in inflammatory zone (FIZZ), consists of four members in mouse (RELM α /FIZZ1/HIMF, RELM β /FIZZ2, Resistin/FIZZ3, and RELM γ /FIZZ4) and two members in human (resistin and RELM β). The importance of these proteins in many aspects of physiology and pathophysiology, especially inflammatory processes, is rapidly evolving in the literature, and many investigators are beginning to work in this field. Most published studies focus on only one isoform, do not evaluate other isoforms that might be present, and have not tested for the specificity of the antibody used. Because RELM isoforms have high sequence and structural similarity and both distinct and overlapping functions, it is important to use a specific antibody to distinguish each isoform in the study. We constructed and established HEK 293 cell lines that constitutively express each isoform. Using these cell lines, we determined the specificity of antibodies (both commercially available and laboratory-made) to each isoform by Western blot and immunofluorescence. Some of the antibodies showed specificity in Western blotting but were not applicable in immunofluorescence. Others showed cross reactivity in Western blot assays. Our results indicate that RELM antibody specificity should be taken into account when using them in research and interpreting data obtained with them.

Keywords

RELM α ; RELM β ; RELM γ ; resistin; antibody specificity

INTRODUCTION

Proteins that comprise the resistin-like molecule (RELM) family are characterized by a unique cysteine-rich motif of C-X11-C-X8-C-X-C-X3-CX10-C-X-C-X-C-X9-C-C at the C-terminus (Banerjee and Lazar 2001; Chumakov et al. 2004; Kim et al. 2001; Stepan et al. 2001b). To date, four isoforms of RELM have been identified in mouse and rat, and two have been identified in human. The nomenclature for this family of proteins is confusing

because each member was discovered independently by separate, unrelated labs as a different functional protein, and each discovery led to a different name being assigned to the same protein family. Thus, the proteins each have multiple names. They have been identified as “found in inflammatory zone” (FIZZ) in an airway reactivity model (Holcomb et al. 2000), as “resistin” and “resistin-like molecule” alpha, beta, and gamma (RELM α , RELM β , and RELM γ) (Gerstmayer et al. 2003; Yang et al. 2003), as “hypoxia-induced mitogenic factor (HIMF)” in a model of pulmonary hypertension (Teng et al. 2003), as adipokines (resistin) (Steppan et al. 2001a; Steppan et al. 2001b), as insulin resistance proteins (ADSF) (Kim et al. 2001), and as chemokines (XCP) (Chumakov et al. 2004). The diverse nomenclature of the four murine and two human isoforms is shown in Table 1. Throughout this article, we will refer to the isoforms as RELM α , RELM β , resistin, and RELM γ .

Proteins in the RELM family have a secretory signal peptide at the N-terminus and a unique, fully conserved motif of 10 equally spaced cysteines at the C-terminus, which form five disulfide bonds. Both human and rodent variants contain between 105 and 114 amino acids, with notable interspecies (36%–56%) and intraspecies (37%–72%) homology (Table 2), especially at the cysteine-rich C-terminus. Murine RELM γ and RELM α have 72% similarity in their amino acid sequence, the highest among all family members (Table 2). RELM γ and RELM β also have high similarity, especially at the C-terminus. In mouse, the genes of RELM α , β , and γ are aligned sequentially on chromosome 16B5 as Retnlb, Retnla, and Retnlg; resistin is located separately on chromosome 8A1. In human, resistin is located on chromosome 19p13.2 and RELM β on chromosome 3q13.1.

Members of the RELM family were initially reported to have distinct tissue distribution (Chumakov et al. 2004; Gerstmayer et al. 2003; Holcomb et al. 2000; Steppan et al. 2001b), and to some extent this holds true. However, this view is changing rapidly as more studies of RELM proteins are published. Under normal physiologic conditions, human resistin expression is seen predominantly in bone marrow, monocytes, and leukocytes, whereas human RELM β is found primarily in colon and small intestine and to a lesser extent in testis and spleen (Chumakov et al. 2004; Nohira et al. 2004). Under normal conditions in mouse, RELM α expresses predominantly in lung, bone marrow, and spleen (Holcomb et al. 2000; Liu et al. 2004; Steppan et al. 2001b; Teng et al. 2002); RELM β expresses in intestinal epithelial cells (Artis et al. 2004; He et al. 2003; Steppan et al. 2001b); resistin expresses in white adipose tissue (Holcomb et al. 2000; Steppan et al. 2001a); and RELM γ expresses in hematopoietic tissues and lung (Chumakov et al. 2004; Gerstmayer et al. 2003). Exceptions to these initial findings have been found, particularly under pathophysiologic conditions and during development, and more are likely to be discovered. For example, both human resistin and RELM β have been reported to be present in the lungs of humans with asthma and scleroderma (Angelini et al. 2009; Homer 2007; Mishra et al. 2007). In the model of helminth-induced Th2-type immunity, murine (m) RELM α , mRELM β , and mRELM γ expression was induced de novo in the lung and liver, where granulomas and inflammation formed after the mice were sensitized and challenged with *S. mansoni* eggs (Pesce et al. 2009). We have found RELM α , RELM β , and RELM γ expression in rodent models of allergic inflammation and in chronic hypoxia models of pulmonary hypertension (unpublished observations).

The study of RELM proteins in physiology and disease is now beginning to expand rapidly. As yet, little is known regarding the differences and similarities in function of the different isoforms. However, most studies of mouse or human RELM have focused on only one RELM isoform and have not evaluated expression of other isoforms in the same experiment. Furthermore, use of in situ hybridization to distinguish the isoforms in tissue is difficult because of the small size of the expressed sequence and the high homology between isoforms. Considering the high sequence similarity among RELM proteins and the growing evidence that multiple isoforms may be expressed within the same tissue, knowledge of antibody specificity is very important for evaluating the distinct expression and functions of each isoform.

Excellent reviews have been published on the issue of antibody specificity, and researchers have offered suggestions on choosing an antibody and on appropriate use of controls when antibodies are used for immunohistology (Couchman 2009; Saper 2005). However, the approach to antibody use is still a complex issue and can be especially difficult when one is studying proteins that have a high degree of similarity, like those in the RELM family. To our knowledge, no one has tested available antibodies for specificity among the different RELM isoforms. Most companies that sell RELM antibodies or ELISA kits have not tested and do not report data for specificity. To help advance the field of RELM protein research, we used cells that express specific isoforms of RELM to test the specificity of RELM antibodies from multiple sources and to provide solid and comprehensive data that researchers can use to choose the most appropriate antibodies.

METHODS

RELM-expressing cell lines

All RELM constructs that we used express fusion proteins that contain a FLAG tag at the C-terminus. We sequenced the DNA of all RELM constructs before transfection to confirm their nucleotide consistency with the NCBI database. In brief, we first inserted PCR products of RELM cDNAs into TOPO-TA vector (Invitrogen, Carlsbad, CA) and then added a FLAG tag sequence to the RELM sequence by inserting cDNAs into pFLAG-CMV 5.1 plasmid (Sigma) using designed restriction sites. Finally, using FLAG-tag primer, we constructed PCR products into pcDNA5/FRT/TOPO TA vector. The positive orientation of constructs was confirmed by sequencing. To establish stable RELM-expressing cell lines, we introduced pcDNA5/FRT-based RELM constructs into Flp-In™ T-REx™ 293 cells (Invitrogen), a modified HEK 293 cell line that we used as host cells. Tetracycline (1 µg/ml) was used to induce expression of recombinant RELMs in the host cells, which were maintained in DMEM supplemented with 5% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml hygromycin B, and 5.0 µg/ml blasticidin.

Antibodies

Mouse anti-FLAG antibody was obtained from Sigma. The RELM antibodies used in this study are from multiple sources. The details for each antibody are listed in Table 3.

Western Blots

RELM-containing medium was collected 2 days after tetracycline induction. Because human (h) RELM β is not secreted, we lysed hRELM β -producing cells in RIPA buffer to obtain that isoform. Thirty microliters of cell culture medium (or 30 μ g of hRELM β cell lysate) were mixed with Laemmli sample buffer (Laemmli 1970) with β -mercaptoethanol and heated for 5 min at 99°C for denaturation. After heating, the samples were subjected to electrophoresis in a 4–20% polyacrylamide gel (BioRad, Hercules, CA). Protein was then electrophoretically transferred onto nitrocellulose membrane (BioRad). The membrane was probed with RELM antibody (1:1000) or anti-FLAG (1:3000, Sigma, St. Louis, MO). The blots were developed with enhanced chemiluminescence (Amersham, Piscataway, NJ) and exposed to X-ray film (Denville Scientific, Metuchen, NJ).

Immunohistochemistry

RELM-expressing HEK 293 cells grown on glass coverslips were induced overnight with tetracycline and then fixed with 4% paraformaldehyde for 10 min. After being washed twice with PBS, the cells were permeabilized with 0.2% Triton X-100 in PBS and then blocked with 2% BSA in PBS before being incubated with anti-RELM antibodies overnight at 4°C. To enhance antigenicity of the protein, in some experiments we treated cells with 0.5% sodium dodecyl sulfate (SDS) before the blocking step, according to published methods (Robinson and Vandre 2001). In other experiments, we treated cells with 2-mercaptoethanol for 3 h at room temperature followed by iodoacetic acid (Campbell et al. 1999) before subjecting them to the SDS treatment. Cy2- or Cy5-conjugated donkey anti-goat IgG and Cy5-conjugated donkey antirabbit IgG were used as secondary antibodies. Nuclei were stained with 50 ng/ml 4',6'-diamidine-2-phenylindole dilactate (DAPI) for 5 min. A Zeiss 510 Meta confocal microscope was used for the imaging. Antibodies to mouse RELM isoforms were tested in all four murine RELM-expressing HEK 293 cell lines, and antibodies to human RELM isoforms were tested in human RELM-expressing HEK 293 cell lines.

RESULTS

mRELM α antibody

Goat anti-mRELM α from R&D Systems (Minneapolis, MN) specifically recognized mRELM α , but no other isoform, by both Western blot (Fig. 1A) and immunohistochemistry (Fig. 1C). Rabbit anti-mRELM α made in our own laboratory also showed good specificity in Western blotting (Fig. 1B).

mRELM β antibody

In addition to recognizing mRELM β , mRELM β antibodies from Peprtech Inc. and Affinity Bioreagents (ABR; Golden CO) also bound weakly to mRELM γ when the antibodies were produced in rabbit by using the full-length mRELM β protein as antigen (Fig. 2A, B). An antibody (kindly provided by Dr. Gary Wu, University of Pennsylvania) produced by using an epitope at the N-terminus after the signal peptide (He et al. 2003) specifically bound to mRELM β on Western blots (Fig. 2C). When its specificity was tested in

immunohistochemistry, mRELM β antibody from ABR recognized mRELM β as well as mRELM γ (Fig. 2D). To enhance antigenicity in immunohistochemistry, we treated the cells with 0.5% SDS for 10 min after they were permeabilized with Triton X-100 and then carried out blocking and antibody incubation. We found that this treatment greatly increased the specificity of the mRELM β antibody provided by Dr. Wu (Fig. 2E).

mResistin antibody

Rabbit anti-mResistin antibody from LifeSpan Biosciences (Seattle, WA) specifically bound to mResistin on Western blots. It also recognized only mResistin by immunohistochemistry (Fig. 3). Although LifeSpan Biosciences claims that this antibody can be used for hResistin, it barely recognized hResistin on Western blot, and it bound equally to hResistin and hRELM β in HEK 293 cells (data not shown). Therefore, this antibody should not be used in human studies of resistin.

mRELM γ antibody

Of the isoforms, mRELM γ has highest homology to mRELM α (72%), so identifying a unique epitope to produce antibody is difficult. Anti-mRELM γ from Alpha Diagnostic International (ADI; San Antonio, TX) produced in rabbit to an unknown peptide recognized both mRELM γ and mRELM α on Western blots (Fig. 4A). We produced mRELM γ antibodies using two different epitopes. When the laboratory-made anti-mRELM γ antibodies were used for Western blotting, one had good specificity for mRELM γ , whereas the other also showed weak binding to mRELM β (Fig. 4B, C). In the immunohistochemical assay, the latter antibody bound all of the mRELM isoforms (Fig. 4D), and the former bound none (data not shown). However, after cells were treated with SDS, the antibody that had initially shown no binding, exhibited binding only to mRELM β protein (Fig. 4E).

hResistin antibody

We tested two hResistin antibodies in our system. Anti-hResistin from Santa Cruz Biotechnology (Santa Cruz, CA) bound weakly to hResistin but recognized mRELM β better. Goat anti-hResistin from R&D Systems specifically recognized hResistin in both Western blots and immunohistochemistry. It recognized no other isoforms, including mouse isoforms (Fig. 5).

hRELM β antibody

When used for Western blotting, anti-hRELM β from Acris Antibodies (San Diego, CA), Adipogen (San Diego, CA), and Chemicon (Temecula, CA) each specifically recognized the target protein hRELM β (Fig. 6A, B). However when used for immunohistochemistry, only goat anti-hRELM β from Acris specifically bound to hRELM β in hRELM β -expressing HEK 293 cells (Fig. 6C).

DISCUSSION AND CONCLUSION

Our laboratory was one of the first to study RELM proteins, particularly mRELM α . While expanding our research interest into other RELM isoforms, we found that some antibodies cross reacted to other family members, a problem that would lead to misleading results if not

taken into consideration. To draw attention to this problem, we have tested the specificity of a variety of commercially available antibodies and some antibodies that we produced ourselves. Not all antibodies that can be used for Western blotting can be effectively applied in immunohistochemistry because in Western blotting, the antibody binds to denatured linear protein whereas in immunohistochemistry, the antibody must recognize a folded protein with tertiary structure. Therefore, we tested each antibody in both Western blot and immunohistochemical assays.

Many companies that provide commercial RELM antibodies have not considered the specificity of their antibodies across isoforms and species. Therefore, investigators need to be very cautious when using available antibodies to study RELM effects or expression and when interpreting data related to RELM protein expression. For example, some mRELM β antibodies can recognize both mRELM β and mRELM γ . Although the signal for mRELM γ is weak, such antibodies cannot be used for immunoprecipitation. We hope that this brief technical report can guide researchers in choosing RELM antibodies for their studies. It is important to look for each of the RELM isoforms in any related study rather than a single isoform in isolation. Additionally, investigators should realize that the same or different cell type within a tissue may express different RELM isoforms. We encourage each investigator and manufacturer to evaluate the specificity of the RELM antibodies that they use in the context of their own work.

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Fig. 1. Specificity of mRELM α antibodies in Western blot and immunofluorescence assays. Antibodies from R&D Systems (A) and our laboratory (B) specifically recognized their target protein on Western blots. When used for immunofluorescence, goat anti-mRELM α antibody from R&D Systems bound only to mRELM α protein in HEK 293 cells (C). Mouse anti-FLAG antibody from Sigma was used to indicate the inductive expression of fusion protein in HEK 293 cells. Cy5-conjugated donkey anti-goat IgG was used as secondary antibody (C, purple).

Fig. 2.

Specificity of mRELM β antibodies in Western blot and immunofluorescence assays.

Although antibodies from Peprtech (A) and Affinity Bioreagents (ABR) recognized mRELM β on Western blots, they exhibited weak cross-reactivity to mRELM γ . Rabbit anti-mRELM β from Dr. Gary Wu's laboratory (University of Pennsylvania) exhibited strong specificity for mRELM β (C). (D) When used for immunofluorescence, rabbit anti-mRELM β antibody from ABR recognized mRELM β and mRELM γ equally. (E) Rabbit anti-mRELM β from Dr. Wu's lab exhibited good recognition of its target protein mRELM β after cells were treated with SDS to enhance antigenicity. Mouse anti-FLAG antibody from Sigma was used to indicate the inductive expression of fusion protein in HEK 293 cells. Cy5-conjugated donkey anti-rabbit IgG (D, purple) and Cy2-conjugated donkey anti-rabbit IgG (E, green) were used as secondary antibodies.

Fig. 3. Specificity of mResistin antibody in Western blot and immunofluorescence assays. Rabbit anti-mResistin from LifeSpan BioSciences (LSBio) specifically recognized mResistin when used for Western blotting (A) and immunofluorescence (B). Mouse anti-FLAG antibody from Sigma was used to indicate the inductive expression of fusion protein in HEK 293 cells. Cy3-conjugated donkey anti-rabbit IgG (C, red) was used as secondary antibody.

Fig. 4.

Specificity of mRELMγ antibodies in Western blot and immunofluorescence assays. (A) Rabbit anti-mRELMγ antibody from Alpha Diagnostics Inc. (ADI) recognized both mRELMα and mRELMγ. (B and C) The two anti-mRELMγ antibodies produced in our laboratory against two different peptide antigens showed fair specificity for mRELMγ on Western blot, although one (#1, B) exhibited weak binding to mRELMβ. However, in the immunofluorescence assay, antibody #1 bound all mRELM isoforms (D). After cells were treated with SDS, antibody #2 that had initially shown no binding, exhibited binding only to mRELMβ protein (4E). Mouse anti-FLAG antibody from Sigma was used to indicate the inductive expression of fusion protein in HEK 293 cells. Cy5-conjugated donkey anti-rabbit IgG (D, purple) and Cy2-conjugated donkey anti-rabbit IgG (E, green) were used as secondary antibodies.

Fig. 5.

Specificity of hResistin antibodies in Western blot and immunofluorescence assays. Goat anti-hResistin from R&D Systems specifically recognized hResistin on Western blots (A), whereas rabbit anti-hResistin from Santa Cruz Biotechnology showed a preference for binding to mRELM β and only weak binding to hResistin (B). (C) The anti-hResistin antibody from R&D Systems also exhibited specificity for hResistin in the immunofluorescence assay. Mouse anti-FLAG antibody from Sigma was used to indicate the inductive expression of fusion protein in HEK 293 cells. Cy5-conjugated donkey anti-goat IgG was used as secondary antibody (C, purple).

Fig. 6. Specificity of hRELM β antibodies in Western blot and immunofluorescence assays. In Western blot assays, hRELM β antibodies from Acris (A), Adipogen, and Chemicon (B) were each highly specific for hRELM β when tested against all RELM isoforms, including mouse. (C) Only the goat anti-hRELM β antibody from Acris exhibited binding to hRELM β in the immunofluorescence assay. Mouse anti-FLAG antibody from Sigma was used to indicate the inductive expression of fusion protein in HEK 293 cells. Cy2-conjugated donkey anti-goat IgG was used as secondary antibody (C, green)

Table 1

The nomenclature for RELM/FIZZ/XCP family of proteins

Murine/Rodent Classification	Human Classification
FIZZ1=RELM α =HIMF=XCP2	Resistin=FIZZ3=XCP1
FIZZ2=RELM β =XCP3	RELM β =FIZZ2=XCP2
FIZZ3=RESISTIN=ADSF=XCP4	
FIZZ4=RELM γ =XCP1	

ADSF, adipose tissue-specific secretory factor; FIZZ, found in inflammatory zone; HIMF, hypoxia-induced mitogenic factor; RELM, resistin-like molecule; XCP, ten-cysteine protein

Table 2

Homology of amino acid sequences among FIZZ/RELM family members in mouse and human

	mRELMα	mRELMβ	mResistin	mRELMγ
mRELM α	100%	48%	37%	72%
mRELM β	48%	100%	42%	62%
mResistin	37%	42%	100%	41%
mRELM γ	72%	62%	41%	100%
hResistin	36%	48%	55%	40%
hRELM β	50%	56%	47%	54%

FIZZ, found in inflammatory zone; RELM, resistin-like molecule

Table 3

Summary of RELM antibody specificity

Target protein	Source	Antigen	Origin	Application	
				Western Blot	Immunohistochemistry
mRELM α	R&D Systems	Full length	Goat	X ^a	X
mRELM α	Dr. Roger Johns' lab, JHU	Peptide (32–46) ENKVKELLANPANYP	Rabbit	X	N ^b
mRELM β	Peptotech Inc.	Full length	Rabbit	mRELM β & weak mRELM γ	N
mRELM β	Dr. Gary Wu U. Penn.	Peptide (28–46) ESLVDQRIKEALSRQEPKT	Rabbit	X	X
mRELM β	Affinity BioReagents, Inc. (ABR)	Full length	Rabbit	X	RELM β & RELM γ
mResistin	LifeSpan BioSciences	Recombinant protein	Rabbit	X	X
mRELM γ	Alpha Diagnostics International Inc. (ADI)	Unknown peptide	Rabbit	mRELM α & mRELM γ	N
mRELM γ	Dr. Roger Johns' lab	Peptide (52–67) TFSCSTISASGRLASC	Rabbit	mRELM γ & weak mRELM β	N
mRELM γ	Dr. Roger Johns' lab	Peptide (32–45) EKKVKELLANRDDC	Rabbit	X	N
hResistin	Santa Cruz Biotechnologies, Inc.	N-terminus 1–70	Rabbit	mRELM β , weak hResistin	N
hResistin	R&D Systems	Full length	Goat	X	X
hRELM β	Acris Antibodies Inc.	Peptide (28–41) DSVMDKKIKDVLNS	Goat	X	X
hRELM β	Adipogen International	Full length	Rabbit	X	N
hRELM β	Chemicon International, Inc.	Unknown	Unknown	X	N

^aX = specificity for target protein^bN = not applicable. JHU, Johns Hopkins University; U. Penn, University of Pennsylvania