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Peripherin-Reactive Antibodies in Mouse, Rabbit, and Human Blood

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Abstract

Type 1 diabetes (T1D) is an autoimmune disorder that results from the destruction of insulin-producing β-cells in the islets of Langerhans. To date, autoimmune T-cell response and antibody reactivity to more than 20 autoantigens have been linked to this disease. Some studies have described the intermediate filament protein peripherin (PRPH) as an autoantigen associated with T1D in non-obese diabetic (NOD) mice. We evaluated immune reactivity of mouse and rabbit sera and human plasma to a 58 kDa protein expressed in RIN-m5F rat insulinoma cells. The protein was isolated using 2-DE and identified by mass spectrometry as PRPH. Antibodies from healthy humans and T1D patients, CD-1 mice, C57BL/6 mice, NOR (non-obese diabetes resistant) mice, and NOD mice reacted with PRPH on Western blots. However, antibody response to PRPH was stronger in NOD than non-autoimmune prone C57BL/6 mice. We conclude that immune reactivity to PRPH is not exclusively associated with NOD mice or human patients with T1D. Furthermore, the frequent occurrence of PRPH-reactive antibodies in mouse and human blood suggests that binding may be non-specific or could reflect the presence of natural autoantibodies against PRPH. These findings point to the need for a re-evaluation of PRPH as a T1D autoantigen in NOD mice and raise the question of the physiological relevance of such widespread immune reactivity against this peripheral nervous system protein.

Keywords

diabetes; autoantibodies; NOD mouse; human; type 1 diabetes

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Supporting Information Available: Supplementary Figures 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

Introduction

Type 1 diabetes (T1D) is an autoimmune disease occurring predominantly in children and youth in which the individual's immune system destroys the insulin producing β-cells. Early prediction of the disease is based mainly on the detection of autoantibodies.1 To date, 24 T1D-related autoantigens have been described, based mostly on the presence of autoantibodies in blood that bind islet proteins.2 In the early 1990s, the intermediate filament protein peripherin (PRPH) was added to the family of T1D associated autoantigens in NOD mice.3–5 Furthermore, a recent study reported that a high proportion of islet infiltrating B lymphocytes produced PRPH reactive antibodies, suggesting that PRPH is a relevant diabetes autoantigen.6

The original aim of the present study was to screen the proteome of diabetes-related tissues for molecular mimics of the diabetes-associated wheat storage globulin homologue of Glb1[7] recently renamed Glo-3A.[8] Western blot analyses using enriched anti-Glo-3A antibodies generated in rabbit revealed a 58 kDa band that was present in the insoluble fraction of rat insulinoma RIN-m5F cells. Subsequent analyses demonstrated that this interaction was not Glo-3A specific. However, a 58 kDa band was also detected with pre-immune rabbit serum and several other antibody preparations. This led us to identify the 58 kDa band as peripherin. We next asked whether peripherin-reactive antibodies were more widespread than previously recognized. These studies are the subject of this report.

Material and Methods

Human Blood

The study was approved by the Ottawa Hospital Research Ethics Board. Clinically proven T1D patient volunteers (females aged 20, 26, 41 y) were recruited through physicians at the Ottawa Hospital, Ottawa, Canada. Unrelated healthy controls were of similar age (male 22, male 28, female 28 y) and of the same Caucasian ethnic group.

Animals

Male C57BL/6 mice at 6–8 weeks of age and CD1 mice at 4 weeks of age were obtained from Charles River Laboratory (Canada). Female NOD weanling mice were obtained from Taconic Farms Inc. (Germantown, NY). Some experiments utilized serum from 7–13 week old NOD, NOR, and C57BL/6 female mice maintained at The Jackson Laboratory (Bar Harbor, ME). The rabbit sera were from Sigma-Genosys (Sigma-Aldrich Canada). Animals were housed under specific pathogen free conditions. The guidance of the Canadian Council on Animal Care for laboratory animals was followed.

Cell Lines

Rat insulinoma cell line RIN-m5F was obtained from the American Type Culture Collection and from Drs. John Mordes and Rita Bortell (University of Massachusetts Medical School). RIN-m5F cells were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, 1 mM sodium pyruvate and 2 mM L-Glutamine. The mouse insulinoma cell line β-TC-6 was provided by Drs. John Mordes and Rita Bortell. β-TC-6 cells were cultured in DMEM medium (Invitrogen) supplemented with 15% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 2 mM L-Glutamine. The mouse neuroblastoma cell line N2a (ATCC No. CCL-131) was provided by Dr. Jagdeep Sandhu (National Research Council, Ottawa, Canada) and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-Glutamine.

Cell Lysis

Frozen cell pellets were homogenized in 100–200 μL lysis buffer (50 mM HEPES pH 7.0, 150 mM NaCl, 0.2% [w/v] CHAPS, 2 μg/mL RNase, 10 U/mL DNase, 2 mM PMSF, 3 μg/mL aprotenin) per 10^6 cells, incubated on ice for 20 min (vortexed every 3–5 min), sonicated in an ultrasonic bath (Bransonic 221) for 5 min and incubated on ice for 10 min. The homogenate was centrifuged at 20 000× *g* at 4 °C for 30 min, the supernatant (cytosolic fraction) was transferred into a new tube and protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad). Pellets were suspended in urea/thiourea buffer [7 M urea, 2 M thiourea, 60 mM DTT and 0.002% bromophenol blue] with volume/pellet equal to lysis buffer. Homogenates were incubated at RT for 30 min and centrifuged at 20 000× *g* at 20 °C for 30 min. The supernatant (membrane fraction) was transferred into a new tube and the pellet was discarded.

Subcellular Proteome Extraction

Subcellular proteome extraction from 6×10^7 RIN-m5F cells or 10^8 N2a cells was performed with ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) according to the manufacturer's protocol for adherent cells. The extraction resulted in four subcellular protein fractions from cytosol (F1), membrane/organelles (F2), nucleus (F3), and cytoskeleton (F4).

SDS-PAGE and Western Immunoblotting

SDS-PAGE and Western immunoblotting were performed as described previously.[9] Briefly, protein samples were diluted in lithium dodecyl sulfate (LDS) sample buffer (4×) (Invitrogen) and heated for 10 min at 70 °C. Subsequently protein samples were separated on 4–12% gradient Bis-Tris NuPAGE 1 mm gels using the NuPAGE electrophoresis system (Invitrogen) and electrotransferred onto 0.45 μm nitrocellulose (NC) membrane (Bio-Rad). After blocking with 5% skim milk (for mouse sera) or 5% human serum albumin (for human plasma) in PBS/Tween-20 at RT for 1 h, the membrane was incubated at RT for 2 h with primary antibody diluted in blocking buffer. Following this, the membrane was washed with PBS containing 0.1% Tween-20 (Fisher Scientific) 3 × 5 min and incubated with the appropriate peroxidase-conjugated secondary antibody at RT for 45 min: rabbit anti-mouse total Ig (DAKO, 1:10 000), goat anti-rabbit total Ig (DAKO, 1:20 000) or goat anti-human (1:20 000) IgG (Fc specific, Sigma-Aldrich). The secondary antibodies alone showed no reactivity with the 58 kDa band. Bands were visualized with the ECL substrate (2.5 mM Luminol, 0.4 mM p-coumaric acid, 0.09% [v/v] H_2O_2, 100 mM Tris-HCl pH 8.0) and exposed to Hyperfilm ECL (Amersham Biosciences).

Two-Dimensional Electrophoresis (2-DE)

According to the manufacturer, the cytoskeleton fraction (F4) cannot be used directly for 2-DE. Therefore a cleanup step was performed prior to 2-DE protein separation. The protein sample was diluted 1:5 with 6 M urea and transferred into an Amicon Ultra-15 30K Centrifugal Filter Device (Millipore) and centrifuged at 750× *g* for 15 min. The flow through was discarded and 10 mL of 6 M urea were added to the concentrate (0.5–1 mL) followed by centrifugation (step performed twice). The concentrate was transferred into a 1.5 mL tube and the protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad). Isoelectric focusing (IEF) was performed with 7 cm ZOOM Strips pH 5.3–6.3 (Invitrogen). For each strip 5 μg protein of F4 fraction were used. The samples were adjusted to 155 μL with rehydration buffer (8 M urea, 2% [w/v] CHAPS, 0.5% [v/v] carrier ampholytes 3–10, 20 mM DTT and 0.002% [w/v] bromophenol blue) and loaded into the wells of the ZOOM IPGRunner cassette. Rehydration was performed at RT for 1 h or overnight at 4 °C. IEF was performed in the ZOOM IPGRunner Cell using the ZOOM Dual

Power supply (Invitrogen) as follows: 30 min/175 V; 90 min/175 – 2000 V ramp; 120 min/2000 V. After equilibration of the strips in sample reducing solution and alkylation solution, SDS-PAGE (second dimension) was performed using 4–12% gradient IPGwell Bis-Tris NuPAGE 1 mm gels. Subsequently, the gels were immunoblotted as described above or silver stained using a mass spectrometry (MS) compatible non-fixing staining protocol. In brief, gels were washed with ddH_2O 3 × 5 min, incubated in 50% ethanol and 5% acetic acid for 30 min and washed for 10 min with 50% ethanol and 10 min with ddH_2O. Next, gels were incubated in 0.02% sodium thiosulphate for 2 min, washed with ddH_2O 2 × 2 min and placed in 0.1% silver nitrate solution for 30 min. Gels were washed in ddH_2O for 1 min and developed using 0.04% formalin and 2% sodium carbonate solution. The reaction was stopped with 5% acetic acid solution.

Mass Spectrometry

The isolated immunoreactive protein spots from 2-DE gels were submitted for LC–MS/MS and MALDI-QTOF-MS/MS to the Ottawa Institute of Systems Biology (OISB, Canada) and the StemCore Laboratories (Ottawa Hospital Research Institute, Canada), respectively. All mass spectra acquired were analyzed and matched to all rat protein sequences in the NCBI database using Mascot (http://www.matrixscience.com) and ProteinProspector (http://prospector.ucsf.edu/prospector/4.27.1/mshome.htm) search engines.

Immunofluorescence Staining

Pancreas sections of 5 μm were cut and attached to charged slides. Immunofluorescence staining and image acquisition were performed as described previously.[10] In brief, pancreas tissue was incubated with guinea pig anti-swine insulin (DAKO) [1:25] and rabbit antiperipherin polyclonal Ab (Chemicon) [1:250] at 4 °C overnight, washed and incubated with biotin-conjugated rabbit anti-mouse (DAKO) Ab [1:300] at RT for 30 min. Thereafter, sections were incubated with streptavidin-conjugated Cy3 (Jackson Lab) [1:400] and goat anti-rabbit/Alexa488 (Invitrogen) [1:400] for 30 min. Nuclei were stained with Hoechst (Sigma) for 1 min. Images were acquired with a Zeiss 510 Meta CLSM microscope using a ×40/1.3 oil Plan-Apochromat objective.

Results

Antibodies in Rabbit Serum Bind to a 58 kDa Protein in RIN-m5F Rat Insulinoma Cells

While screening the proteome of β-TC-6 and RIN-m5F insulinoma cells for molecular mimics of Glo-3A, we found pre-immune rabbit serum contained antibodies that reacted with a 58 kDa protein that was abundantly present in the insoluble fraction of RIN-m5F cells but absent in β-TC-6 cells (Figure 1A). Several other unrelated immune sera also showed binding to the 58 kDa protein (data not shown).

To determine the subcellular localization of the 58 kDa protein we used a ProteoExtract kit (Calbiochem) to extract the proteins from RIN-m5F cells according to their subcellular location. The four fractions (F1–F4) were screened for the presence of the 58 kDa protein using SDS-PAGE and Western blot analysis. We detected the protein of interest mainly in the cytoskeleton fraction (Figure 1B).

Identification of the 58 kDa Protein by Mass Spectrometry

To identify the isoelectric point (p*I*) of the 58 kDa protein we performed 2-DE with the protein extract obtained from the insoluble pellet (membrane fraction) of lysed RIN-m5F cells using wide range IEF strips. We detected three spots of 58 kDa migrating in the p*I* range of 5 to 6 on Western blots (data not shown). For the isolation and identification of the 58 kDa protein we used the cytoskeleton fraction (F4) of RIN-m5F cells obtained from the

subcellular proteome extraction. As seen in the Commassie blue stained gel, this fraction contained a relatively smaller number of proteins (Figure 2A) enabling a better separation of the specific protein spots on 2-DE gels. Because the protein of interest had a p*I* of ~5.6 we used strips with a narrower pH range of 5.3–6.3 to enhance the resolution. Four strips were loaded with 5 μg/strip of total protein. After IEF in the first dimension, proteins were separated by SDS-PAGE in the second dimension. One gel was silver stained. Proteins from the remaining three gels were transferred onto nitrocellulose membranes. Western blots were performed using pre-immune rabbit serum, CD1 mouse serum, and NOD mouse serum. All three Western blots showed an identical spot pattern. We could clearly match the spots on the silver stained gel with the 58 kDa spots detected by Western blots (Figure 2B). To identify the corresponding proteins, the spots were excised individually and subjected to QTOF-MALDI MS analyses. All three samples matched the expected MS profile of rat peripherin (Table I and Figure 1S, Supporting Information) with scores >10[11] (ProteinProspector).

We then used the cytoskeleton fraction of the RIN-m5F cells to screen various mouse sera and human plasma for reactivity to the 58 kDa protein. Of the samples we tested, all interacted with the 58 kDa protein to varying degrees (Figure 3). All of the NOD mouse sera displayed strong reactivity to the 58 kDa band. Sera from C57BL/6, CD1 and NOR mice displayed mixed reactivity ranging from weak/moderate to strong. Although the band was weaker than in NOD mice, antibodies were also present in plasma from three patients with longstanding T1D as well as plasma from healthy control subjects. Because antibody reactivity to the 58 kDa protein was present in sera from autoimmune diabetes prone NOD mice as well as control strains, we wanted to more rigorously test whether there were quantitative differences. Sera pooled from 4–5 NOD, NOR, and C57BL/6 female mice at 7–12 weeks of age were used at different dilutions for Western blot analyses to compare the antibody reactivity to the 58 kDa protein. As shown in Figure 3B, when sera from non-autoimmune prone C57BL/6 mice were diluted to 1:400, antibody reactivity against the 58 kDa protein was nearly lost. In contrast, serum from NOD and the closely related NOR strain continued to demonstrate antibody reactivity against the 58 kDa protein until reaching a dilution of at least 1:1600. The NOR strain shares ~88% of its genome with NOD including many T1D susceptibility loci. While NOR mice do not progress to overt T1D, they do develop insulitis indicating the presence of some level of autoimmune responses.[11] These results indicate that while the presence of antibodies against the 58 kDa band was not diabetes-specific, immune reactivity was stronger in the presence of genes contributing to the development of autoimmunity.

Evaluation of Peripherin Expression in Insulinoma and Neuroblastoma Cell Lines

As shown in Figure 1A, the 58 kDa band was detected in RIN-m5F cells but not in β-TC-6 cells. This suggested the rat insulinoma cell line expressed PRPH while the mouse insulinoma cell line did not. To further confirm this, we used an anti-PRPH rabbit serum to evaluate the expression of PRPH in these insulinoma cell lines. As expected, a strong signal was detected at 58 kDa in the membrane fraction of RIN-m5F cells and no signal was observed in the β-TC-6 cell extracts (Figure 4). These results confirmed that the 58 kDa band detected with the various sera was the intermediate filament protein PRPH.

To determine whether this immune reaction was also seen with mouse PRPH, we used the N2a mouse neuroblastoma cell line which expresses PRPH.[12] The subcellular proteome of the N2a cells was extracted using the subcellular proteome extraction kit. The four fractions obtained were separated by SDS-PAGE and analyzed on Western blot using anti-PRPH serum which confirmed PRPH expression in N2a cells (Figure 5A). We then selected randomly C57BL/6 and NOD mouse sera and performed Western blot analysis with N2a nuclear and cytoskeleton protein fractions. All samples reacted with a 58 kDa protein in the

cytoskeleton fraction (Figure 5B) confirming the cross-reactivity of the antibodies with mouse PRPH. As further confirmation of specificity, we found that sera from C57BL/6, NOR and NOD mice bound to purified, recombinant mouse PRPH on Western blots (data not shown). When mouse islets were probed with anti-PRPH serum, staining was observed in neuroinsular complexes and around the periphery of islets (Figure 2S, Supporting Information). However, PRPH staining did not colocalize with insulin^{+} cells.

Discussion

The measurement of autoantibodies remains the primary approach to evaluate the activation of β-cell autoimmunity in humans[13] and NOD mice.14 Some of the major autoantigens include: insulin,15 glutamic acid decarboxylase,16 IA-2,17 islet specific glucose-6-phosphate catalytic subunit related protein (IGRP),[18] and zinc transporter ZnT8 (Slc30A8). 19 In 1992, PRPH was also proposed as a T1D autoantigen in NOD mice.5 These authors reported the presence of autoreactive anti-PRPH antibodies in sera from NOD mice but not in control sera from wild type mice. Furthermore, a recent study reported that islets from mouse strains developing insulitis contain B lymphocytes that secrete PRPH specific autoantibodies.6 On the basis of this finding, the authors concluded that PRPH is a relevant autoantigen in diabetogenesis.

In the present study, using the ECL detection method, which is more sensitive than the previously used colorimetric detection,[5] we show the presence of PRPH-reactive antibodies in all evaluated wild type and NOD mouse sera, as well as human T1D patient and healthy control plasma. To our knowledge, this is the first report of PRPH reactive antibodies in humans and wild type mice. We did not observe differences in the reactivity of T1D patients compared with healthy controls. In contrast, the reactivity of C57BL/6, CD1 and NOR mice sera against PRPH was less pronounced than in NOD mice. However, antibody reactivity to PRPH was not found exclusively in NOD mice[5] or in patients with T1D.

The fact that we detected immune reactivity to PRPH in all sera of mice and humans raises the possibility that antibodies reactive with PRPH could be natural autoantibodies. The immune response against PRPH was elevated in diabetes-prone NOD mice and diabetes resistant NOR, two strains that display autoimmune responses. The term natural autoantibody defines self-protein reactive antibodies that are detectable in the absence of immunization with the target antigen (reviewed in refs 20 and 21). Natural autoantibodies are important for the maintenance of homeostasis and participate in the clearance of proteins released from senescent or lysed cells. For example, antibodies directed against the cytoskeleton proteins actin and tubulin are considered to be natural autoantibodies.22,23 Under normal conditions these cytoskeleton proteins are not accessible by natural autoantibodies because they are not exposed to the extracellular environment. Thus, this process of autoimmunity is similar to recognition and clearance of foreign antigens. PRPH is a type III intermediate filament cytoskeleton protein that is mainly expressed in the peripheral neurons.[3,24,25] Therefore, natural auto-antibodies directed against this protein could contribute to the process of clearance of senescent or dying peripheral neurons.

In conclusion, we demonstrate that PRPH-reactive antibodies are present in wild type CD-1, C57BL/6, NOR and diabetes-prone NOD mice as well as healthy subjects and T1D patients. Therefore, the presence of PRPH-reactive antibodies is not associated exclusively with NOD mice or patients with diabetes. These findings do not rule out possible involvement of PRPH in diabetogenesis. However, they demonstrate that PRPH-reactive antibodies are not exclusively linked with diabetes and occur more frequently than previously recognized. Two of the coauthors (DVS, HDC) have preliminary evidence that will be the subject of a future manuscript which indicates transgenically increasing the frequency of peripherin reactive B-

lymphocytes accelerates the rate of autoimmune diabetes development in NOD mice. Therefore, it will be important to re-evaluate what role PRPH plays in diabetogenesis, particularly in humans and to investigate the physiological relevance of such widespread immune reactivity against this molecule.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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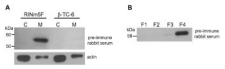

Figure 1.

Immune reactive 58 kDa cytoskeleton protein in RIN-m5F rat insulinoma cells. (A) Cytosolic, C and membrane, M proteins (20 μg/lane) of rat RIN-m5F and mouse β-TC-6 insulinoma cells were separated by SDS-PAGE on a 4–12% gel and probed by Western blot with rabbit pre-immune serum [1:300]. The serum reacted with a 58 kDa protein present in the membrane fraction of rat insulinoma cells. β-actin was probed with antibodies as a loading control. (B) Cytosolic (F1), membrane/organelle (F2), nuclear (F3) and cytoskeleton (F4) proteins of RIN-m5F cells were separated and an immunoblot was performed as described above. The 58 kDa protein of interest was present mainly in the cytoskeleton fraction.

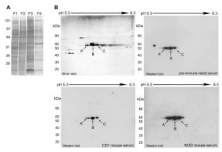

Figure 2.

Detection and isolation of the 58 kDa protein using 2-DE Western blot and silver stain analysis of the cytoskeleton fraction of the RIN-m5F cells. (A) Cytosolic (F1), membrane/organelles (F2), nuclear (F3) and cytoskeleton (F4) proteins of the RIN-m5F cells were separated in a 4–12% gel and stained with GelCode blue (Pierce). The image shows a low number of proteins in the cytoskeleton fraction compared with the other three fractions. (B) Cytoskeleton proteins (5 μg/strip) were separated using IEF (pH 5.3–6.3 strips) and followed by SDS-PAGE (4–12% gels). Four 58 kDa spots were detected between p*I* 5.5–5.7 by Western blot using pre-immune rabbit serum [1:200], CD1 mouse serum [1:100] and NOD mouse serum [1:100]. The spots could be clearly matched to four spots on a silver stained gel. The spots were excised (first two spots were excised as one sample) and subjected to MS analysis.

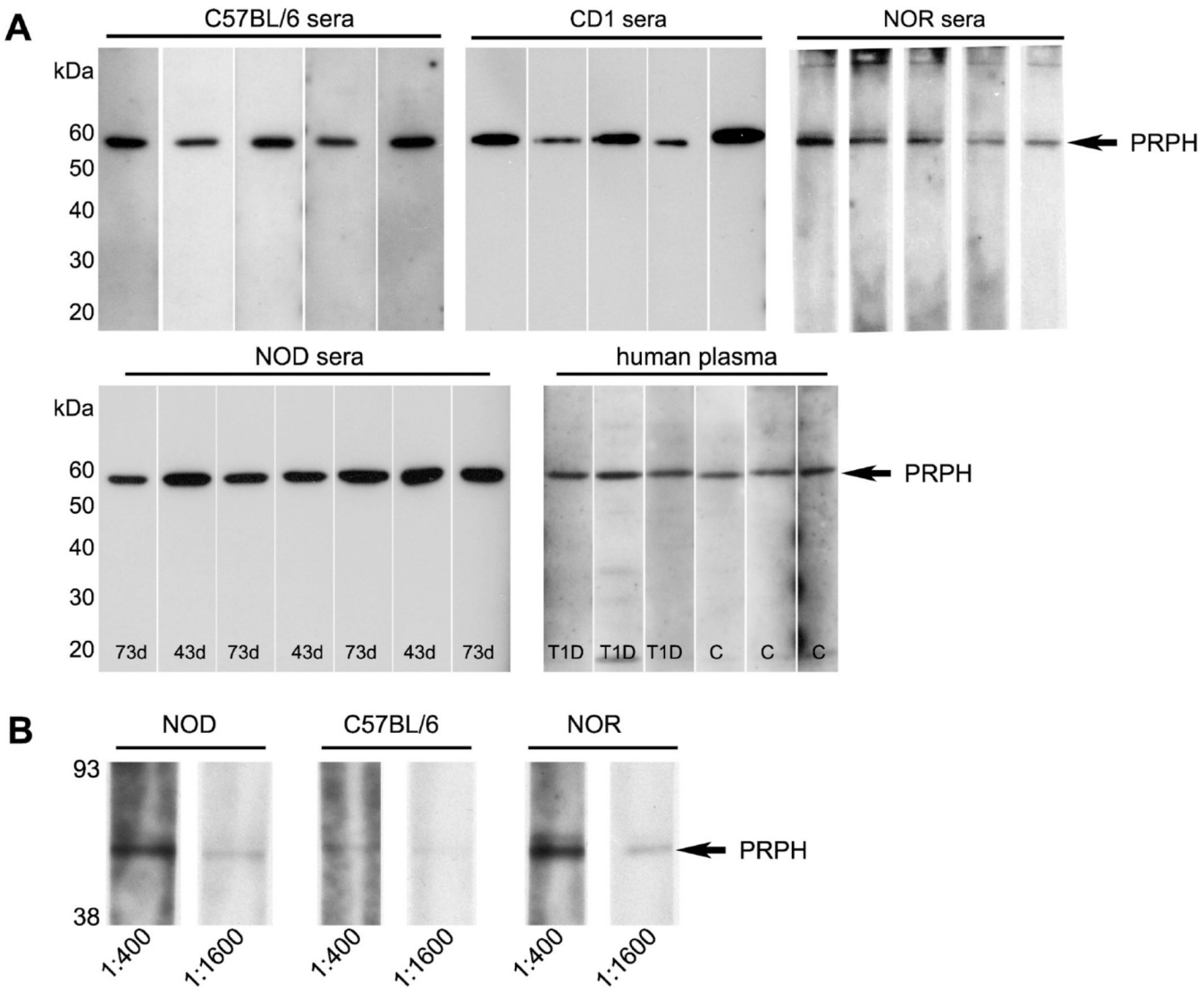

Figure 3.

Antibody reactivity against the 58 kDa protein is present in mouse sera and human plasma. (A) Proteins of the cytoskeleton fraction (~1 μg protein/lane for NOR sera and 4.25 μg protein/lane for all other sera) were separated on 4–12% gels and Western blots were performed using C57BL/6, CD1, NOR and NOD mouse sera [1:100], and human plasma from T1D patients and healthy control, C subjects [1:100]. (B) Sera from 4–5 NOD, NOR, and C57BL/6 female mice at 7–12 weeks of age were assessed at dilutions of 1:400, and 1:1600 in Western blot analyses for antibody reactivity against the 58 kDa protein in the cytoskeleton fraction of RIN-m5F cells.

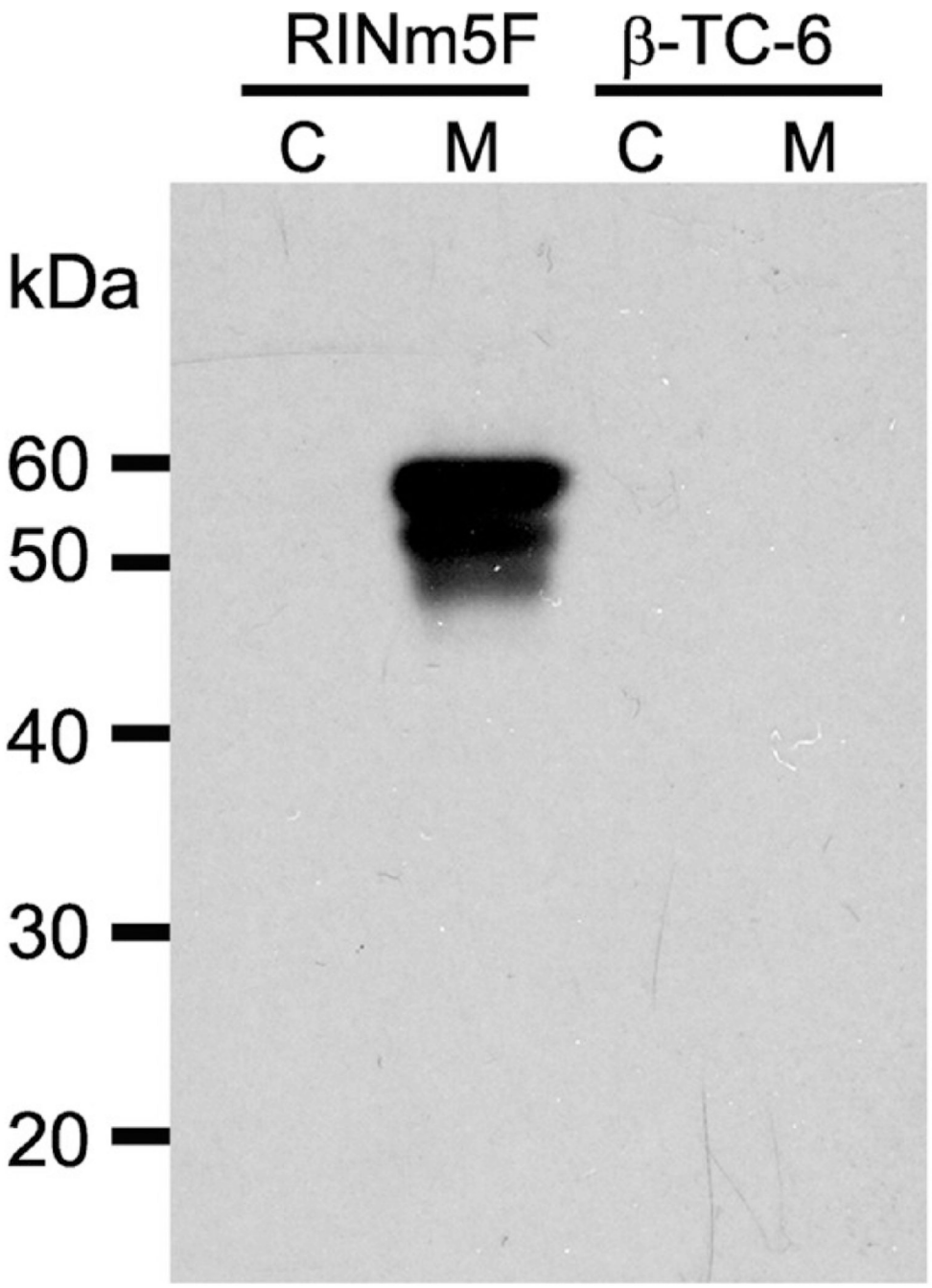

Figure 4.
Intermediate filament protein PRPH expression in rat and mouse insulinoma cell lines. Cytosolic (C) and membrane (M) proteins (20 μg/well) of RIN-m5F rat insulinoma cells and β-TC-6 mouse insulinoma cells were separated using SDS-PAGE on a 4–12% gel. The separated proteins were transferred onto nitrocellulose membrane and probed in Western blot with a rabbit anti-PRPH serum [1:20 000].

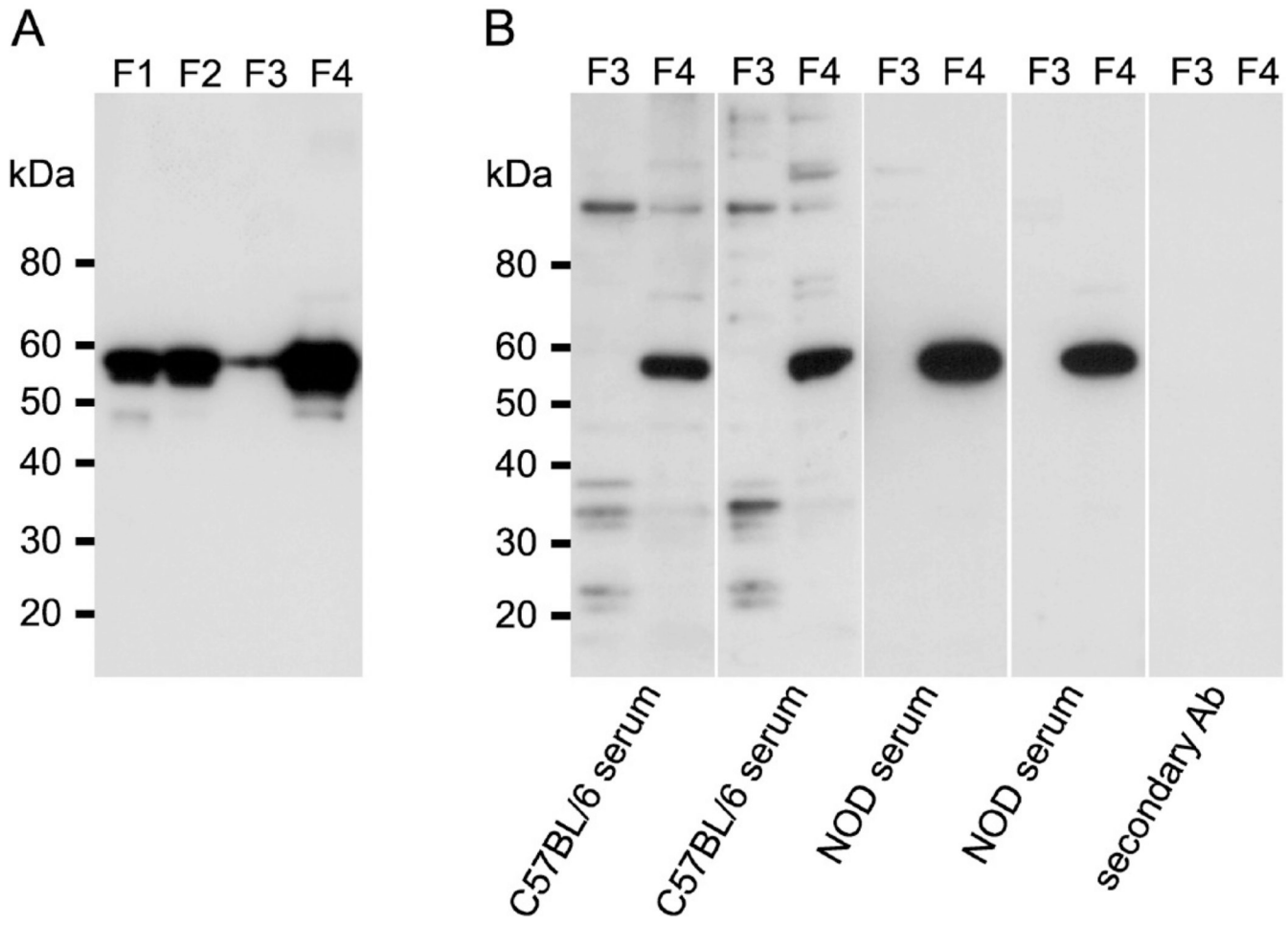

Figure 5.

Antibodies from control and diabetes-prone mice react with mouse PRPH. (A) To confirm PRPH expression in N2a mouse neuroblastoma cells cytosolic (F1), membrane/organelles (F2), nuclear (F3), and cytoskeleton (F4) proteins of N2a cells were separated by SDS-PAGE and aWestern blot was performed using a rabbit anti-PRPH serum [1:40 000]. (B) Fractions F3 and F4 of N2a cells were separated by SDS-PAGE and probed by Western blot with C57BL/6 and NOD mouse sera [1:100]. For the negative control, protein extracts were incubated with the secondary HRP conjugated antimouse Ig Ab only [1:10 000].

Table I

Identification of the 58 kDa Protein as Peripherin using MALDI-QTOF MS[a]

spot	score	identification	accession no.	kDa/p*I*		peptide matches (*n*)	sequence coverage (%)
				theoretical	observed		
A	3.7×10^{12}	peripherin	EDL87004	57.6/5.5	58/5.5	30	45.5
B	6.2×10^{14}	peripherin	EDL87004	57.6/5.5	58/5.6	34	57.7
C	7.5×10^{11}	peripherin	EDL87004	57.6/5.5	58/5.7	28	44.5

[a]The results were obtained by sequence database searches with ProteinProspector software using the parameters described before.[9]