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Identification of GRASP-1 as a novel 97 kDa autoantigen localized to endosomes

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Abstract

We have identified an autoantigen that is recognized by antibodies from an 18-year-old female with a history of recurrent infections who later in her clinical course developed Raynaud's phenomenon and telangiectasias. By indirect immunofluorescence (IIF), the index serum produced a unique cytoplasmic discrete speckled (CDS) staining pattern that partially colocalized with early endosome antigen 1 (EEA1) but not Golgi complex or other cytoplasmic organelles in HEp-2 cells. When HEp-2 cells were treated with 0.1 N HCl, the cytoplasmic speckled staining of the index serum was markedly decreased, suggesting that the reactive antigen was soluble. Western blot analysis showed a reactive ~97 kDa protein in a saline soluble protein preparation from HeLa cells. Mass spectrometric analysis of the excised 97 kDa band that was immunoprecipitated from HeLa cell extracts identified GRASP-1 as a possible target. The index serum and anti-GRASP-1 antibodies colocalized to structures in the cytoplasm of HEp-2 cells. Synthetic peptides representing the full-length GRASP-1 protein were used to identify reactive epitopes. Like many other cytoplasmic autoantigens, GRASP-1 has numerous coiled-coil domains throughout the protein with the exception of short segments at the amino and carboxyl terminus.

Keywords: Autoantibodies; Autoimmunity; Endosomes; GRASP-1; Coiled-coils

Introduction

Autoantibodies are important serological markers of autoimmune diseases and have previously been used as reagents to successfully isolate and characterize a number of cellular antigens. For example, novel nuclear [1,2], Golgi complex [3,4], mitochondrial [5,6], ribosomal [7], and endosomal [8,9] antigens have been identified using human autoantibodies.

Several autoantigens have been identified in the endosomal trafficking pathway. They include early endosome antigen 1 (EEA1) [9,10], lysobisphosphatidic acid (LBPA) [11], and cytoplasmic linker protein-170 (CLIP 170) [12]. EEA1 is a hydrophilic peripheral membrane protein associated with the cytoplasmic side of early endosomes [13,14]. Autoantibodies to EEA1 have been found in patients with neurological diseases, subacute cutaneous lupus [9,10], Raynaud's phenomenon, Wegener's granulomatosis, and proteinuria [15]. LBPA is an anionic phospholipid enriching the internal membranes of late endosomes [16]. Anti-LBPA antibodies are present in the plasma of some patients with anti-phospholipid antibodies (APLAs), which are associated with thrombosis and/or recurrent pregnancy loss [17,18]. CLIP-170 is a member of the cytoplasmic linker proteins that facilitate the interaction of cellular organelles, such as endosomes, to microtubules. CLIP-170 was localized to the plus ends of microtubules,

Abbreviations: cANCA, cytoplasmic anti-neutrophil cytoplasmic antibodies; CLIP, cytoplasmic linker protein; EEA1, early endosome antigen 1; ELISA, enzyme linked immunosorbent assay; GRASP-1, glutamate receptor interacting protein-associated protein-1; GW/GWB, glycine (G) and tryptophan (W) rich proteins localized in distinct cytoplasmic foci referred to as GW bodies; Hrs, hepatocyte growth factor receptor substrate: LBPA, lysobisphosphatidic acid; MS, mass spectroscopy.

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binds to newly polymerized tubulin, and is an activator of the microtubule-based motor, dynactin [19–22]. Thus far, autoantibodies to CLIP-170 have only been found in patients with systemic sclerosis, glioblastoma, and idiopathic pleural effusion [12].

Autoantibodies to lysosomes are characterized as a large irregular speckled staining pattern distributed throughout the cytoplasm [23,24]. Except for the well-characterized antigens in neutrophils characterized as cANCA [25,26], lysosome autoantigens have not been extensively characterized or understood. One such autoantigen was identified as the glycoprotein h-lamp-2 in the cytoplasm of neutrophils [27]. H-lamp-2 is a transmembrane protein with extensive glycosylation. Anti-h-lamp-2 antibodies were identified in the sera of patients with necrotizing and crescentric glomerulonephritis (NCGN). It was found that 14 out of 16 patients with NCGN and anti-neutrophil cytoplasmic antibodies contained antibodies to h-lamp-2 [27].

In this study, our attention focused on a serum that produced a unique cytoplasmic staining pattern in HEp2 cells and reacted with an unidentified protein in immunoblots of tissue culture cell extracts. The index serum was from an 18-year-old female with a history of recurrent infections and a presumed immune deficiency who later developed Raynaud's phenomenon and telangiectasias. Based on mass spectrometry, colocalization, and epitope mapping studies, we have identified the target autoantigen as GRASP-1.

Materials and methods

Human sera and patients

The index serum was from an 18-year-old female with a history of recurrent infections and a presumed immune deficiency who later developed Raynaud's phenomenon and telangiectasias. Other human sera used in this study were obtained from the Advanced Diagnostics Laboratory, University of Calgary. Control sera were collected from healthy volunteers or randomly selected from a bank of 2000 blood donors [28]. The serum samples were stored at −20°C or −70°C.

Indirect immunofluorescence (IIF) and ELISA

IIF was performed on commercially prepared HEp-2 cells (Immuno Concepts Inc., Sacramento, CA) using a fluorescein (FITC)-conjugated affinity-purified goat anti-human IgA + IgG + IgM (H + L) (Jackson Immuno Research, West Grove, PA) as previously described [29–31].

Colocalization

Colocalization studies used affinity-purified Cy3™-conjugated donkey anti-mouse IgG (H + L) (Jackson Immuno Research) or affinity-purified Cy3™-conjugated donkey anti-rabbit IgG (H + L) (Jackson Immuno Research) and the appropriate primary antibodies. Primary polyclonal antibodies raised in rabbits included: EEA1 [9], Hrs (hepatocyte growth factor receptor substrate) (a gift from Dr. E.K.L. Chan, University of Florida, Gainesville, USA), and GRASP-1 (a gift from Dr. R Huganir, The John Hopkins University School of Medicine, Baltimore, Maryland). Murine monoclonal antibodies were: EEA1 (Transduction Laboratories, Mississauga, ON), golgin-97 (CytoStore, Calgary, AB), LAMP2 (lysosome associated membrane protein-2; a gift from J. August and J. Hildreth, The John Hopkins University).

Acid extraction

Saline-soluble proteins were extracted from HEp-2 cells (ImmunoConcepts Inc.) by immersion of slides in 0.1 N HCl for 30 min at room temperature and then washing them in phosphate-buffered saline (PBS) for 10 min followed by IIF as previously described [32]. Human sera with anti-histone [32] and anti-GW antibodies [33] were used as controls. The slides were overlayed with a cover slip using Vectashield® mounting media (Vector Laboratories Inc., Burlingame, CA) that contained 4′,6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei. The slides were then viewed with a Leica DMRXA2 confocal microscope with Chroma HiQ filter sets. Images were taken with a Princeton Instrument Inc. digital camera, and Adobe Photoshop (version 6.0) was used to process the images.

ELISA

A commercially available EIA Kit (anti-phospholipid-8Pro-G, Alpco Diagnostics, Windham, NH) was used to test for the presence of antibodies directed against β2-glycoprotein I, cardiolipin, cardiolipin combined with β2-glycoprotein I in the same well, phosphatidyl–choline, phosphatidyl–ethanolamine, phosphatidyl–inositol, phosphatidyl–serine, and sphingomyelin according to the manufacturer's instructions. The reactivities were read at an absorbance of 450 nm on a Biomek 1000 (Beckman Coulter Canada Inc., Mississauga, ON).

Cell lines and extracts

HeLa cells (ATTC CCl 2.2; American Type Culture Collection, Rockville, MD) were grown in Dulbecco's Modified Eagle Media (DMEM, Gibco) supplemented with 10% FCS, 2 mmol/l L-glutamine, and 1% penicillin–streptomycin. Cells were grown to confluence, lysed by adding Buffer A (150 mM NaCl, 10 mM Tris pH 7.5, 1.5 mM $MgCl_2$, 0.5% NP-40) for 5 min, scraped from the flask and centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was collected and stored at −70°C until required. The protein concentrations of the homogenates

were determined by a Protein Assay Reagent Kit (Pierce, Rockford, IL).

SDS-PAGE and immunoblotting

The HeLa cell saline-soluble protein preparations described above were solubilized in SDS sample buffer, separated by discontinuous SDS-PAGE [34], and transferred to nitrocellulose as previously described [35]. Nitrocellulose strips were blocked with 5% non-fat milk in PBS-T (0.05% Tween-20), overlaid with the primary antibody for 1 h, and then washed in 3 changes of PBS-T for 10 min each. Bound antibody was traced with goat anti-human immunoglobulin conjugated to horseradish peroxidase (Calbiochem-Behring Corp., La Jolla, CA), and identified by adding enhanced chemiluminescence (ECL) substrate solution (Amersham Life Science Ltd, Aylesbury, UK). Reactive proteins on the nitrocellulose strips were visualized by exposure to and recording on X-OMAT AR film (Eastman Kodak Co., Rochester, NY).

Immunoprecipitation and mass spectrometry

HeLa cell extracts prepared as described above were combined with 400 μl NET2+F buffer (20 ml NP-40, 35.06 g NaCl, 40 ml 0.5 M EDTA, 200 ml 1 M pH 7.4 Tris, 20 ml 20% SDS, 800 mg sodium azide, 20 g deoxycholic acid in 4 l distilled water), 50 μl suspension of anti-human IgM (μ-chain specific) agarose (Sigma), and 10 μl of serum for 2 h at 4°C on a rotator. Unbound protein was washed away with five washes in NET2+F buffer, and the bound proteins were analyzed by 10% SDS-PAGE [34]. The gels were stained with silver (SilverQuest™, Silver Staining Kit, Invitrogen), and unique bands were excised and sent for analysis to the Southern Alberta Mass Spectrometry Centre (University of Calgary, AB) for matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) using a trypsin digestion protocol. MS data were analyzed using ProFound (http://prowl.rockefeller.edu/cgi-bin/ProFound) and MoverZ software to process the spectra (http://prowl.rockefeller.edu/software/mz.htm).

GRASP-1 epitope mapping

Epitope mapping employed sequential peptides of 15 amino acids offset by five amino acids, representing the full-length GRASP-1 protein, which were synthesized on membranes using the SPOT technology as previously described [36–38]. The membranes containing the peptides were processed for immunoblotting by soaking the membrane in Tris-buffered saline (TBS; 10 mM Tris–HCl pH 7.6, 150 mM NaCl) for 10 min and then blocking with 2% milk/TBS for 1 h at room temperature. The human sera were diluted 1/100 in 2% milk/TBS and applied to the membrane. After 2 h of incubation at room temperature, the membrane was washed three times with TBS. A horseradish-peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch) was diluted according to the manufacturer's protocol, and reactivity was visualized using enhanced chemiluminescence Western blotting detection reagents (Amersham International) and recording on X-OMAT AR film (Eastman Kodak Co.). To determine if other proteins shared epitopes with GRASP-1, the sequences of reactive peptides were submitted for BLAST analysis on the NCBI server. The deduced protein sequence was analyzed for coiled-coil motifs with the COILS algorithm [39] on the Swiss EMBNet server (http://www.ch.embnet.org/software/COILS).

Results

Indirect immunofluorescence and ELISA

The index human serum (LA) was characterized by IIF as cytoplasmic discrete speckled in HEp-2 cells with a titre of >1:3200 (Fig. 1). The reactive antigenic structures were heterogeneous in size and tended to be evenly dispersed throughout the cytoplasm but not all cells had the same number of dots. The number varied from 0 to 30. The structures were observed throughout the cell cycle, including metaphase cells (see Fig. 1).

Colocalization studies showed that the index human serum did not colocalize with a marker for the *trans*-Golgi compartment, golgin-97 (Fig. 2A), with LAMP2 (Fig. 2B), a marker for late lysosomes, or with Hrs (Fig. 2C), a marker for endosomes. However, the reactive antigen demonstrated partial overlap and colocalization with rabbit anti-EEA1 (Fig. 2E), but not the murine monoclonal anti-EEA1 (Fig. 2D).

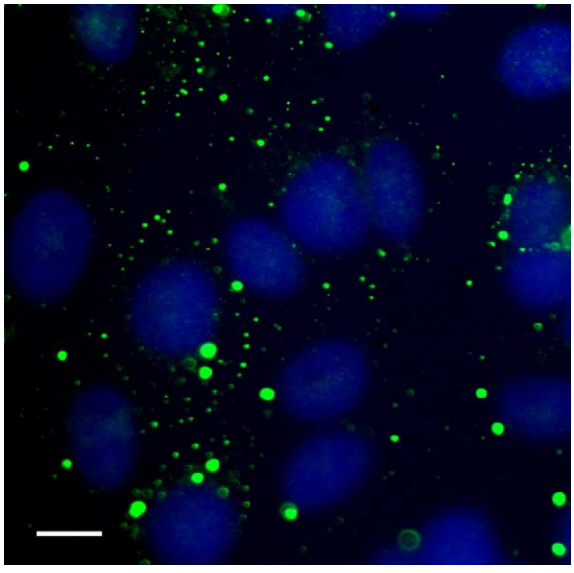

Fig. 1. The index serum produces a cytoplasmic discrete speckled (CDS) pattern of staining on HEp-2 cells. The structures range in size from 0.1–1.0 mm in diameter, are present in cells throughout the cell cycle, and number as many as 30 in some cells but are not found in others. Nuclei are stained with DAPI. The scale bar line represents 10 μm.

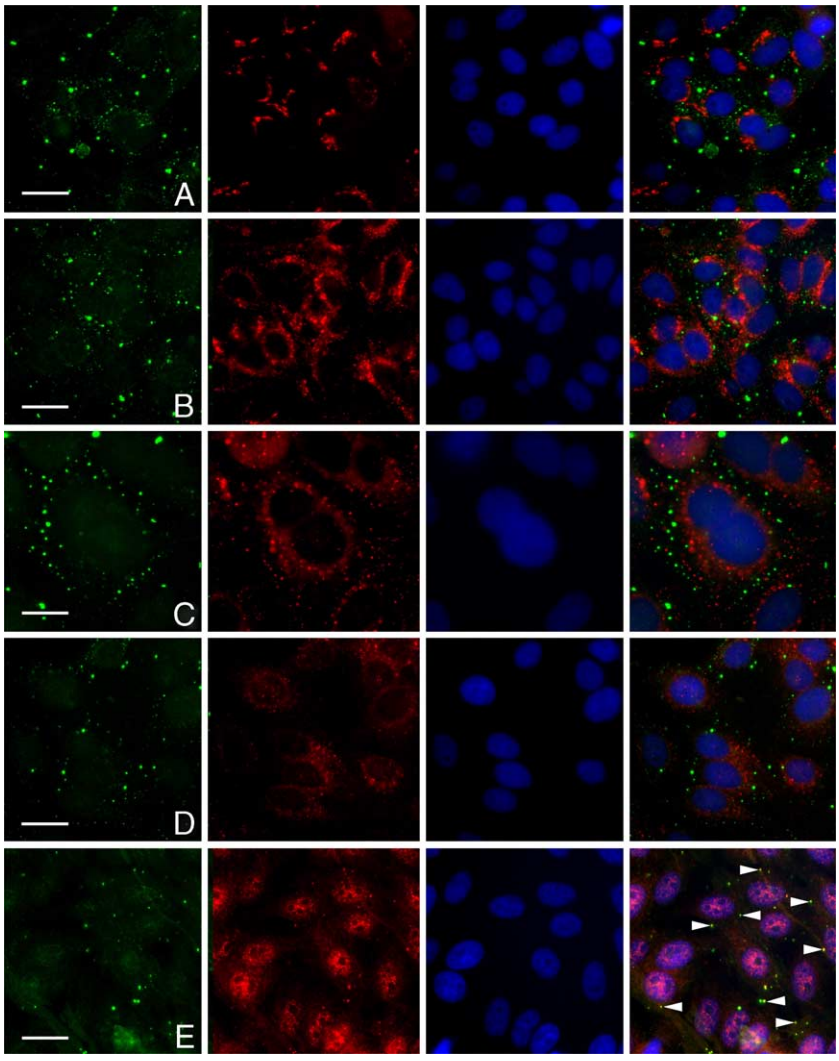

Fig. 2. Cytoplasmic structures identified by the index serum are distinct from known cytoplasmic compartments in HEp-2 cells. The first column represents staining with the index human serum at 1/80 dilution using as a secondary goat anti-human immunoglobulin conjugated to FITC. The second column represents several different cytoplasmic markers: (A) mouse monoclonal anti-golgin-97 antibody, (B) anti-LAMP2 antibody, (C) anti-Hrs antibody, (D) mouse monoclonal anti-EEA1 antibody, (E) rabbit anti-EEA1 antibody. The third column shows corresponding DAPI-stained cell nuclei. The fourth column represents images merged from the three columns to the left. The index serum colocalizes with some endosome vesicles marked by the rabbit anti-EEA1 antibody (arrows, right panel E). The scale bar line represents 10 μm.

The index serum did not react with β2-glycoprotein I, cardiolipin, cardiolipin combined with β2-glycoprotein I in the same well, phosphatidyl–choline, phosphatidyl–ethanolamine, phosphatidyl–inositol, phosphatidyl–serine, or sphingomyelin (data not shown).

Acid extraction

Treatment of cells with dilute acid typically removes all saline-soluble proteins (i.e. Sm, histone) from the cell [32]. When HEp-2 cells were treated with 0.1 N HCl, the reactive antigen was extracted or non-reactive, and the cytoplasmic speckled staining produced by the index serum became negative (Fig. 3A). Reactivity of a control serum with antibodies to GW bodies under the same conditions was not affected by the acid treatment (Fig. 3B), while the staining pattern of a serum containing anti-histone antibodies [32] was markedly reduced as expected (Fig. 3C). These observations suggested that the reactive antigen is soluble.

The reactive antigen is GRASP-1

Reactivity with a 97-kDa protein was noted when the index serum was used to immunoblot the soluble HeLa cell extracts (Fig. 4). Immunoprecipitation of HeLa-soluble proteins with the index serum followed by mass spectrometry analysis of the excised ~97 kDa band identified a positive candidate antigen as GRIP-associated protein 1 (GRASP-1) (expectation of 0.015). Further support for the conclusion that the reactive antigen is GRASP-1 was shown by colocalizing studies of the index

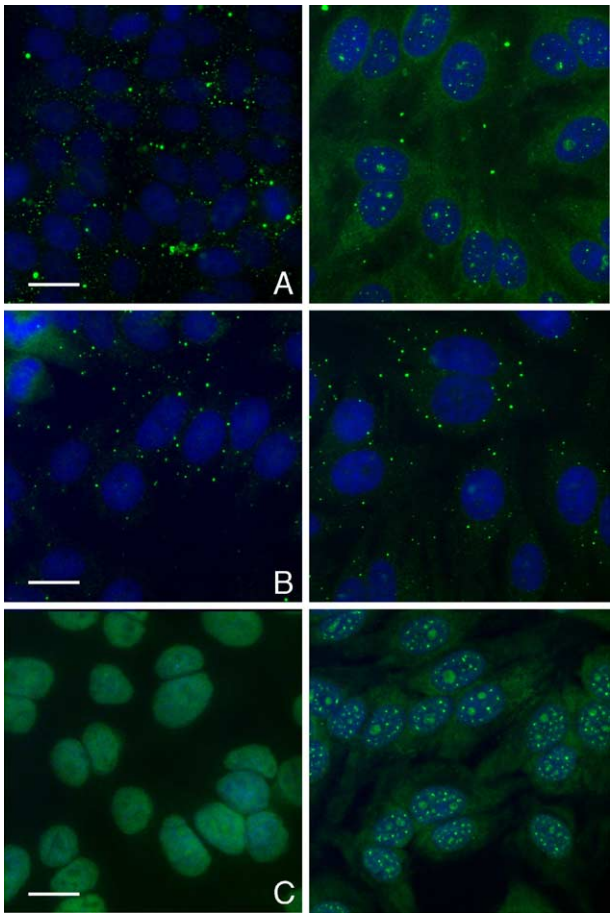

Fig. 3. IIF on HEp-2 cells before (left column) and after (right column) treatment of cells with 0.1 N HCl. The CDS pattern of staining is abolished after extraction (A), whereas GW bodies stained by a human anti-GWB sera remain intact (B). A human serum antibodies to histones and nuclear matrix proteins show that the homogeneous nuclear staining is removed, leaving insoluble nuclear matrix proteins behind (C). Nuclei are stained with DAPI. The scale bar line represents 10 μm.

human serum with polyclonal anti-GRASP-1 (Fig. 5). In these studies, the majority of large cytoplasmic dots of the index serum were found to colocalize with the GRASP-1 cytoplasmic antibodies. Other more diffuse cytoplasmic staining produced by both antibodies did not appear to colocalize, suggesting that both proteins are also found in other cytoplasmic pools.

GRASP-1 epitope mapping

Multiple 15 mer peptides over the length of GRASP-1 were recognized by the index serum (Fig. 6, Table 1). The reactive epitopes were not concentrated in any specific domain. When the reactive pentapeptides were subjected to a BLAST analysis, several proteins showed 100% amino acid sequence identity. Of interest, the amino sequence EKLKK is a peptide found in the 70 kDa heat shock protein. The pentapeptide DLKRQ was identified in rabaptin-5, and the peptide RDLVK is found in component X of the pyruvate dehydrogenase complex (PDC; Table 2).

Discussion

Historically, autoantibodies to nuclear antigens have been the primary focus in systemic rheumatic diseases [40,41]. However, in the past decade, more attention has been given to cytoplasmic antibodies [42]. Among these are antibodies localized in Golgi complex [29], mitochondria [5], ribosome [7], endosome [8,9], and lysosomes [27]. These cytoplasmic antigens have different subcellular localizations and diverse functions.

The purpose of this study was to identify another unique cytoplasmic autoantigen. Colocalization studies showed that the index serum reacted with an antigen that showed partial colocalization with EEA1 of endosomes in HEp-2 cells. Immunoprecipitation of HeLa cell extracts and mass spectrometry identified GRASP-1 (glutamate receptor interacting protein (GRIP)-associated protein-1) as the target antibody. Additional studies, including colocalization and epitope mapping, confirmed that the index serum recognizes GRASP-1.

GRASP-1 was identified as a protein that binds to the seventh PDZ domain of GRIP (glutamate receptor interacting protein) [43]. GRIP is a PDZ (postsynaptic synaptic density-95/discs large/zona occludens-1) domain containing protein that serves as an adaptor protein and links AMPA glutamate receptors to other proteins in a macromolecular signal-transducing complex. AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate) glutamate receptors mediate rapid excitatory synaptic transmission critical in the induction and maintenance of learning and memory [43]. GRIP is involved in mechanisms that are implicated in the targeting and clustering of AMPA receptors [44]. Additionally, GRIP was charac-

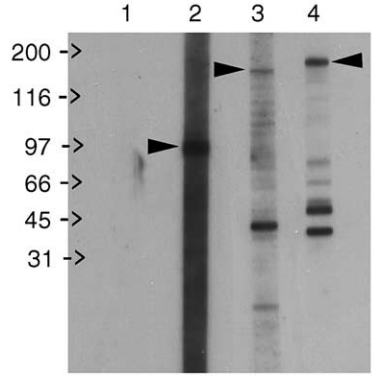

Fig. 4. Immunoblot of HeLa soluble cell extract detected a cellular protein of ~97 kDa (arrow head) specifically recognized by the index human serum (lane 2). Human anti-EEA1 identify the expected ~160 kDa protein (arrow head: lane 3) and anti-GW182 identified a ~180 kDa protein (arrow head: lane 4). Normal human serum (lane 1) is the negative control.

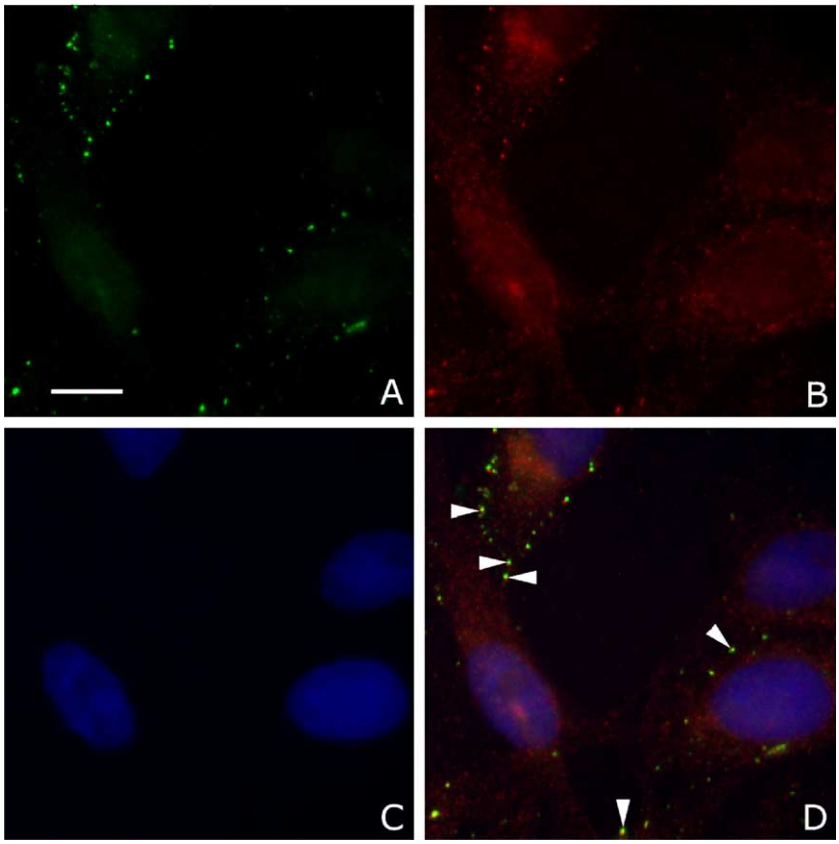

Fig. 5. Immunofluorescence analysis of HEp-2 cells by costaining of rabbit anti-GRASP-1 antibody and the index human serum. (A) Index human serum at 1/80 dilution, (B) rabbit anti-GRASP-1 antibody at 1/200 dilution, (C) nuclei counterstained with DAPI, (D) colocalized and merged images of GRASP-1 and structures marked by the index serum appearing yellow (arrows). The scale bar line represents 10 μm.

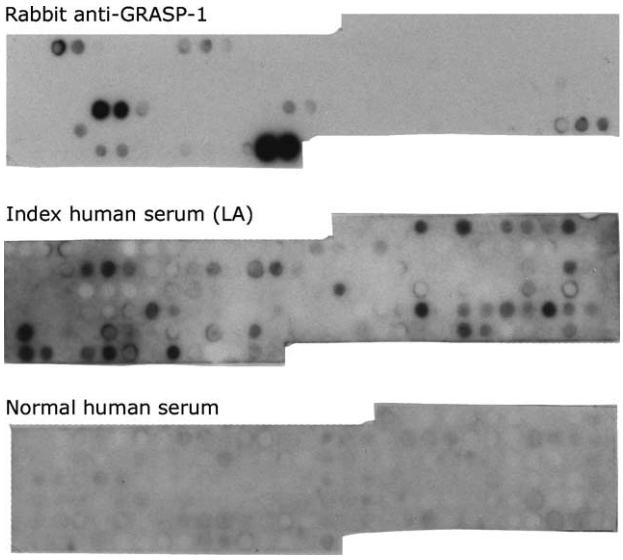

Fig. 6. Epitope mapping obtained using sequential 15 mer peptides offset by 5 amino acids that represented the full-length GRASP-1 protein were spotted on membranes and then probed with a rabbit polyclonal antibody to GRASP-1, the index serum (LA), and a normal human serum. The rabbit serum binds to a more restricted set of distinct peptides including the C-terminal immunogen (lower left) when compared to the human serum that demonstrates more diverse reactivity with other peptides but not the C-terminal peptides bound by the rabbit serum.

terized to be involved in the sorting, transport, and possibly recycling of membrane proteins from the cytoplasm to the dendrites and synapses of neurons throughout the brain [45]. GRIP contains seven PDZ domains and therefore simultaneously binds multiple ligands to form a large adaptor complex. GRIP has been shown to interact with ephrine (EPH) receptors, ephrines [46], kinesin heavy chains [47], and several GRASPs [43]. GRASPs were discovered by screening yeast two-hybrids against different PDZ domains of GRIP1. GRASP-1 was found to specifically interact with the seventh PDZ domain of GRIP. Sequence analysis of GRASP-1 revealed a complex domain structure including a *ras*GEF domain in the N-terminal region, an RBD (*ras* binding domain) and PDZ domain in the C-terminal region, and a potential caspase-3 cleavage site in between. GRASP-1 is a neuronal specific guanine nucleotide exchange factor (GEF) of the *ras* family of small G proteins. The small G protein *ras* is involved in a variety of functions including cell growth, differentiation, and transformation. It is unclear as to which *ras* is the in vivo target of GRASP-1 and what this function may be, although previous studies have shown that the recruitment of *ras*GEF to signal transduction complexes plays a role in the activation of G proteins and downstream signal

Table 1
Amino acid sequence and position of the GRASP-1 protein synthetic peptides and their reactivity with a polyclonal rabbit serum, the index serum (LA), and normal human serum (NHS)

GRASP-1 Peptide			Patient		
No.	Sequence	Position	Rabbit Anti-GRASP-1	LA	NHS
4	QLLELRTNNYQLSDE	16 30			
5	RTNNYQLSDELRKNG	21 35			
6	QLSDELRKNGVELTS	26 40			
7	LRKNGVELTSLRQKV	31 45			
8	VELTSLRQKVAYLDK	36 50			
9	LRQKVAYLDKEFSKA	41 55			
10	AYLDKEFSKAQKALS	46 60			
11	EFSKAQKALSKSKKA	51 65			
16	EMLQAKLHSQEEDFR	76 90			
17	KLHSQEEDFRLQNST	81 95			
22	QMEQLEQENQQLKEG	106 120			
23	EQENQQLKEGAAGAG	111 125			
24	QLKEGAAGAGVAQAG	116 130			
45	RLQEELAKLSEKLKK	221 235			
46	LAKLSEKLKKKQESF	226 240			
47	EKLKKKQESFCRLQT	231 245			
51	FNDSRNKIEELQQRK	251 265			
53	LQQRKEADHKAQLAR	261 275			
54	EADHKAQLARTQKLQ	266 280			
67	AENNALRTSLAALEQ	331 345			
85	RKSAEKRKAMLDELA	421 435			
93	LGVRARYERELRELH	461 475			
102	VEELQAQVHSMDGAK	506 520			
103	AQVHSMDGAKGWFER	511 525			
104	MDGAKGWFERRLKEA	516 530			
105	GWFERRLKEAEESLQ	521 535			
110	KQCREQHAAELKGKE	546 560			
111	QHAAELKGKEEELQD	551 565			
116	ERDCHLKTISSLKQE	576 590			
117	LKTISSLKQEVKDTV	581 595			
118	SLKQEVKDTVDGQRI	586 600			
119	VKDTVDGQRILEKKG	591 605			
120	DGQRILEKKGSAALK	596 610			
121	LEKKGSAALKDLKRQ	601 615			
122	SAALKDLKRQLHLER	606 620			
123	DLKRQLHLERKRADK	611 625			
124	LHLERKRADKLQERL	616 630			
125	KRADKLQERLQDILT	621 635			
126	LQERLQDILTNSKSR	626 640			
129	SGLEELVLSEMNSPS	641 655			
130	LVLSEMNSPSRTQTG	646 660			
131	MNSPSRTQTGDSSSI	651 665			
135	REILREKESSAVPAR	671 685			
137	AVPARSLSSSPQAQP	681 695			
146	KVKHLEVSSASMAED	726 740			
147	EVSSASMAEDLCRKS	731 745			
150	AIIETYVMDSRIDVS	746 760			
151	YVMDSRIDVSVAAGH	751 765			
152	RIDVSVAAGHTDRSG	756 770			
154	TDRSGLGSVLRDLVK	766 780			
155	LGSVLRDLVKPGDEN	771 785			
156	RDLVKPGDENLREMN	776 790			
157	PGDENLREMNKKLQN	781 795			
158	LREMNKKLQNMLEEQ	786 800			
159	KKLQNMLEEQLTKNM	791 805			
161	LTKNMHLHKDMEVLS	801 815			
164	QEIVRLSKECVGPPD	816 830			
165	LSKECVGPPDPDLEP	821 835			
166	CVGPPDPDLEPGETS	826 840			

Gradients of white to black shows increasing intensity of reaction of antibodies with the peptide.

Table 2
Representative epitopes and proteins demonstrating complete sequence identity by BLAST analysis

Deduced epitope from Table 1	Corresponding GRASP-1 amino acids	Protein with sequence similarity	GenBank accession	Species
EKLKK	231–235	Heat shock 70 kDa protein 4 isoform a	NP_002145	*Homo sapiens*
DLKRQ	611–615	Rabaptin, RAB GTPase binding effector protein 1	CAI26055	*Mus musculus*
RDLVK	778–782	Pyruvate dehydrogenase complex, E2 component; dihydrolipoamide *S*-acetyltransferase	NP_785657	*Lactobacillus plantarum* WCFS1

transduction [48,49]. Overall, GRASP-1 is part of a large complex of proteins and is thought to be involved in the regulation of AMPA receptor function and play a role in AMPA receptor synaptic targeting.

GRASP-1 is an 110-kDa protein specifically expressed in all tissues of the nervous system (including cortex, cerebellum, hippocampus, olfactory bulb, thalamus, spinal cord, and brainstem) but not in muscle, lung, heart, liver, kidney, or spleen [43]. The cellular distribution of GRASP-1 was expressed in neurons in the soma and dendritic processes, but not the glia. Cells from various brain regions (cortex, hippocampus, thalamus, striatum, and brainstem cerebellum) had GRASP-1 expression. In cortex and hippocampus, GRASP-1 was observed in the pyramidal cells and other neurons; in cerebellum, GRASP-1 was expressed in Purkinje cells and granular cells. The tissue and cellular localization of GRASP-1 is similar to that of GRIP1 and AMPA receptors. Staining of the index serum on cerebellum showed a pattern similar to that of GRASP-1 (results not shown).

The results of the BLAST analysis of reactive epitopes are noteworthy. For example, HSp-70 is a known autoantigen in several diseases including rheumatoid arthritis [50,51], and rapabtin 5 is an effector of the small GTPase Rab5, which is involved in endocytic membrane fusion [52]. This result is also noteworthy because rapabtin 5 is found in the same macromolecular complex with Rab5 and EEA1 [53], the latter being a known target autoantigen [9,10]. This finding is also interesting because the index serum demonstrated partial colocalization with rabbit EEA1. Finally, the reactive pentapeptide RDLVK was found in component X of the pyruvate dehydrogenase complex (PDC). Autoantibodies to PDC are most commonly found in patients with primary biliary cirrhosis (PBC) [54]. Of note, in our mass spectrometry studies with the index serum, the E2 component of PDC was identified as a potential target protein. Immunoblot of the index serum with purified PDC demonstrated reactivity with an unidentified band in this preparation (data not shown).

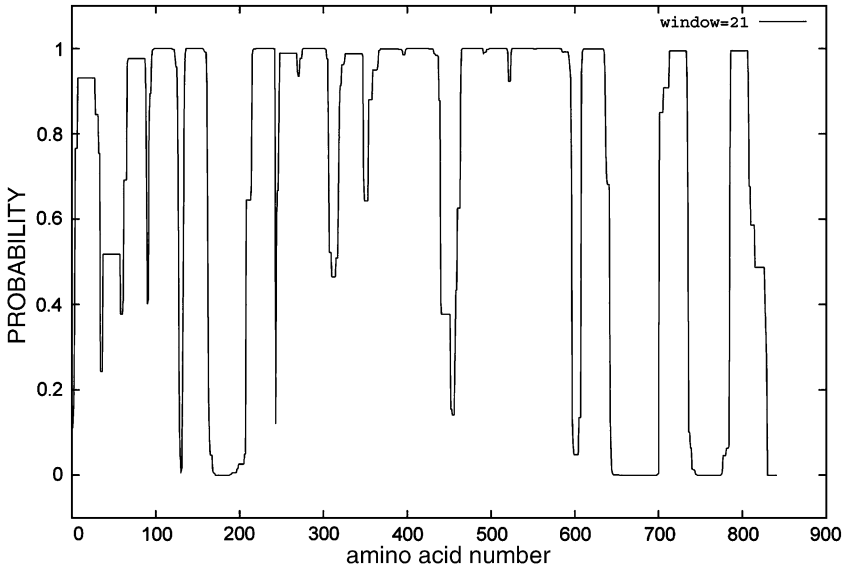

Fig. 7. Probability plots for the determination of coiled-coil domains of human GRASP-1 (Accession # NP 064522). The amino acid numbers are indicated on the abscissa, and the percent probability of a coiled-coil region is indicated on the ordinate. GRASP-1 is depicted as a plot of probability for the formation of coiled-coils calculated by the COILS program [39] (http://www.ch.embnet.org/software/COILS_form.html). GRASP-1 has multiple coiled-coil domains except at the amino and carboxy terminus.

The identification of GRASP-1, an autoantigen characterized by multiple alpha-helical coiled-coil domains (Fig. 7), is consistent with the features of a number of other cytoplasmic autoantigens including all Golgi complex and endosome autoantigens (reviewed in [42,55]) and many others identified to date [42]. The reason proteins characterized by extensive coiled-coil domains appear to be preferred B-cell targets is not fully understood [42].

Conclusion

In summary, we have used serum from an 18-year-old female to identify GRASP-1 as a target autoantigen. The serum reacted with a ~97 kDa saline-soluble autoantigen that produced a unique cytoplasmic dot staining pattern on HEp-2 cells. Mass spectroscopy, colocalization, and epitope mapping studies have confirmed that GRASP-1 is a target recognized by the index serum.

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