BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **229,** 280–286 (1996)
ARTICLE NO. 1793

Evidence for the Formation of Multimeric Forms of the 5A11/HT7 Antigen

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Received October 24, 1996

The 5A11 antigen is the avian homologue of a developmentally regulated 45-kDa glycoprotein of the immunoglobulin super-gene family implicated in heterotypic cell-to-cell interactions. Employing chemical cross-linking agents, we provide evidence for oligomerization of the 5A11 antigen in Triton X-100-solubilized preparations of neural retina, liver, and erythrocytes and in intact erythrocytes. Dimerization was demonstrated through partial proteolytic digestion and diagonal gel electrophoresis. Sedimentation velocity separation demonstrated that the 5A11 dimer, derived from retina, is part of a larger macromolecular complex characterized by a sedimentation rate comparable to that of the glutamine synthetase octomer (S = 15.2). In contrast, the sedimentation velocities of the dimer and monomer from erythrocytes were similar. These data provide the first biochemical evidence of interaction of the 5A11 antigen and differences in these interactions between tissues.

The 5A11 antigen is the chicken homologue of a widely distributed 45 kDa glycoprotein of the IgSF (1,2) also identified as the HT7 antigen (3,4), neurothelin (4,5), basagin (6), the MRC OX-47 antigen (7), the M6 antigen (8), EMMPRIN (9), the CE9 antigen (10), and mouse gp42 (11). Interest in these molecules stems from the independent identification of high level expression during phenotypic maturation of a broad variety of cell types and tissues. We first identified the 5A11 antigen in embryonic chick retina through production of a monoclonal antibody that possessed the capacity to inhibit cell-to-cell interaction-dependent processes *in vitro* (1,12). Recently, a factor called EMMPRIN, identified as the human homologue of the 5A11/HT7 antigen, was demonstrated to be enriched on the surface of human tumor cells and implicated in tumor invasion and metastasis (9).

The developmental distribution, association with morphogenesis (2), and implication in cell interactions (1,12) and tumor metastasis (9) emphasize the need for a thorough understanding of the 5A11 glycoprotein's role in mediating cell-to-cell interactions. In the present study, we have used a combination of chemical cross-linking, non-ionic detergent extraction and sedimentation velocity to demonstrate distinct protein-protein interactions involving the 5A11 antigen.

MATERIALS AND METHODS

Antibodies. The production of the 5A11 and 3B7 Mabs have been described previously (12,13). Other Mabs used in these studies were as previously described (13,14,15,16,17). Polyclonal rabbit antiserum against the 5A11 antigen was generated in our laboratory by subcutaneous immunization and boosting of male New Zealand white rabbits with

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Abbreviations used: kDa, kilodaltons; IgSF, immunoglobulin super gene family; DSS, disuccinimidyl suberate; DSP, dithio-bis-succinimidylpropionate; BS3, bis-sulfosuccinimidyle suberate; DTSSP, 3,3′-dithio-bis-sulfo-succinimidylpropionate; PMSF, phenylmethylsulfonyl fluoride; TBS, tris-buffered saline; NCAM, neural cell adhesion molecule.

0006-291X/96 $18.00

immuno-affinity purified 5A11 antigen (13) and Ribi's adjuvant. Also used was polyclonal rabbit antiserum to chicken glutamine synthetase (18,19). Polyclonal rabbit antiserum to the murine antigen CE9, generously provided by James Bartles (20). Peroxidase-conjugated secondary antibodies were obtained from Boehringer Mannheim, and ^{125}I-labeled secondary antibodies were obtained from New England Nuclear. For several experiments immunoreactivity was visualized with an enhanced chemiluminescence kit from Dupont.

Tissue isolation. Fertilized white leghorn chicken eggs were obtained from the Division of Poultry Science of the University of Florida. Inbred, BalbC mice were killed by cervical dislocation. Tissues were isolated as previously described (13).

Polyacrylamide gel electrophoresis and immunoblotting. One dimension SDS-PAGE, transfer to nylon backed nitrocellulose (MSI) and immunolabeling were as previously described (13). In some experiments, peroxidase-conjugated antibodies were used at a dilution of 1:10,000 and visualized using the chemiluminescence reagent (Dupont) followed by exposure of X-OMAT AR film (Kodak) for 30 sec to 4 hrs.

Two-dimensional diagonal SDS-PAGE was performed by first separating proteins by SDS-PAGE on 7.5% gels under non-reducing conditions. The sample lanes were then excised, equilibrated in sample buffer containing 2% beta-mercaptoethanol and affixed with 1% agarose in stacking gel buffer on top of a second 7.5% gel. Following electrophoresis, gels were fixed in a solution of methanol/acetic acid/water (5:1:5), and the location of polypeptides was determined by silver staining using the Sigma silver staining Kit, or by autoradiography.

Chemical cross-linking. All cross-linking reagents were obtained from Pierce. In the initial experiments, disuccinimidyl suberate (DSS), dithio-bis-succinimidylpropionate (DSP), and bis-sulfosuccinimidyle suberate (BS3) were prepared in DMSO at a concentration of 40 mM. Subsequently, the water-soluble, membrane impermeable agents BS3 and 3,3′-dithio-bis-sulfosuccinimidylpropionate (DTSSP) were dissolved in Tyrode's solution at a concentration of 25 mM. Isolated tissues were solubilized in 4 volumes 1% Triton X-100 in Tyrode's solution. The reagents were added to the solubilized tissue at a final ratio of 1:50-100 (vol:vol) and incubated at 4°C for 15 min-30 min. The reactions were quenched by the addition of primary amines (10 mM glycine or 10 mM tris, pH 7.4 and 1 mM PMSF). Insoluble material was pelleted by centrifugation at 10,000 × g for 15 min at 4°C. The pellet was resuspended by vortexing in buffer containing 1% Triton X-100, 0.5% NP-40 and 0.1% SDS, and insoluble material was removed by centrifugation. An equivalent amount of each sample was mixed with 4× SDS-PAGE sample buffer and incubated at 95°C prior to electrophoresis.

Erythrocytes were resuspended at a concentration of 1×10^7 cells/ml Tyrode's solution in the presence or absence of 0.1% Triton X. BS3 or DTSSP were added at a final concentration of 0.5-2.5 mM. The reactions were quenched by the addition of free primary amines, and detergent concentration in all samples was adjusted to 1%. The detergent-soluble and-insoluble proteins were separated by centrifugation at 10,000 × g for 15 min. The pellets were washed in 1 % Triton X-100 in TBS and centrifuged a second time. The final pellet was resuspended in 1% Triton X-100 containing 0.25 % SDS. Samples were prepared for immunoblotting as described above.

Radio-iodination. Surface proteins were radiolabeled by lactoperoxidase catalyzed iodination. Glucose oxidase (0.1 U/ml; Sigma), lactoperoxidase (1 U/ml; Sigma) and 1 mCi carrier-free ^{125}I (New England Nuclear) were sequentially added to the 1×10^7 cells in 250 ml Tyrodes solution containing 20 mM glucose. After 30 min at 4°C, the reaction was stopped by the addition of 100 mM tyrosine followed by repeated washing of the cells in Tyrodes solution. The iodinated cells were exposed to extracellular crosslinking by BS3 or DTSSP.

Limited proteolysis. The 5A11 antigen was immunoaffinity purified from iodinated erythrocytes as previously described (13). Antigen containing fractions were pooled and desalted (Spectrum; Houston, TX). Cross-linked and non-crosslinked species were separate by SDS-PAGE under non-reducing conditions. Limited proteolysis of polypeptides was performed as described (21) with 2 U of V8 protease (endoproteinase Glu-C; EC 3.4.21.19; Sigma). Such analyses were conducted on at least three separate occasions using labeled protein from different experiments.

Sucrose gradient ultracentrifugation. Detergent solubilized proteins (above) were centrifuged at 35,000 rpm (100,000 × g) at 4°C for 30 min. 200 ml of the high speed supernatant (AF1 mg protein) from each tissue preparation were layered onto a 3.8 ml, 5-20 % linear sucrose gradient prepared in extraction buffer and centrifuged for 13-18 hrs at 100,000 × g in a Beckman SW 50.1 rotor at 4°C. 200 ml fractions were collected from the bottom of each tube. The protein concentration of each fraction was determined using the Biorad Bradford reagent with bovine serum albumin as standard. The refractive index of fractions was determined using a Zeiss refractometer. Equal volumes were used for Western blot analysis or immunodot assay (13,22).

RESULTS

Chemical cross-linking of 5A11 antigen from neural retina, liver, and erythrocyte. In preliminary studies, Western blot analysis of retina exposed to the cross-linking reagent BS3 produced novel and strong immunoreactive bands at 98 kDa, 200 kDa and >200 kDa only faintly observed with the other cross-linking reagents. Therefore, water soluble cross-linking reagents were utilized for the remaining studies.

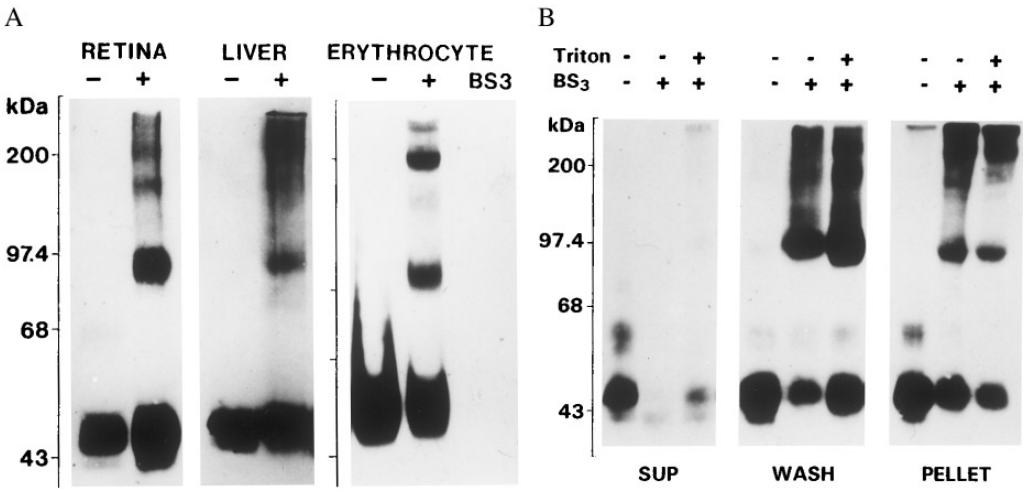

FIG. 1. Chemical cross-linking of the 5A11 antigen in retina, liver, and erythrocyte. (A) Proteins were solubilized in Triton X-100 in the absence (−) or presence of 0.8 mM BS3 (+) followed by immunoblot analysis. In retina, liver, and erythrocyte, a 45.5- to 50-kDa polypeptide was labeled by the 3B7 Mab. Exposure to BS3 resulted in immunolabeling of cross-linked polypeptides at 98, 150, and 200 kDa. (B) Erythrocytes were treated with (+) or without (−) 1.5 mM BS3 in the presence (+) or absence (−) of 0.1% Triton X-100. Detergent-soluble (SUP), wash (WASH), and insoluble proteins (PELLET) were immunoblotted using the 3B7 Mab. 50-kDa polypeptides were labeled in all samples. Chemical cross-linking resulted in prominent immunoreactivity of 98, 150, and 200 kDa in the WASH and the PELLET fractions. The locations of BRL-prestained molecular weight markers are indicated; 200 kDa (myosin heavy chain), 97.4 kDa (phosphorylase B), 68 kDa (bovine serum albumin), and 43 kDa (ovalbumin).

Cross-linking was repeated with retina, liver and erythrocyte extracts. In mock-crosslinked samples, polypeptides of 45-50 kDa were immunolabeled in each tissues (Fig. 1A). Exposure to BS3 generated immunoreactivity at 98 and 200 kDa (Fig. 1A) in all 3. Similar experiments in the mouse, using the CE9 antiserum, resulted in immunolabeling of the novel 98 kDa (data not shown) imply conservation of the interaction across species.

The above experiments were conducted in the presence of the non-ionic detergent Triton X. Thus, the data cannot differentiate potential cell-to-cell interactions from receptor complex formation within the plasma membrane, or interaction with intracellular polypeptides. To resolve these possibilities, experiments were repeated in the absence of detergent using isolated chick erythrocytes and the water soluble, membrane-impermeable reagent BS3.

BS3 treatment in the presence of 0.1% Triton X-100 completely solubilized all cells such that only nuclei were observed under the microscope. In contrast, BS3 treatment in the absence of Triton X, did not lyse nor agglutinate the erythrocytes, rather they remained a single cell population. Western blot analysis, prominent bands of 45 and 55 kDa were observed in the soluble, the wash and the insoluble pellet in each sample (Fig. 1B). The labeling of 2 bands could be accounted for by the lack of reduction of intramolecular disulfide bonds (compare to Fig. 1A). In the presence of BS3, additional staining was observed at 98, 200, and >200 kDa, both with or with out detergent in the cross-linking reaction (Fig. 1B). These bands were localized to the wash and pellet fractions suggesting association with the detergent in-soluble cytoskeleton. More importantly, the data indicate crosslinking of the 5A11 antigen to a polypeptide also defined by an extracellular domain accessible to the membrane-impermeable reagent BS3.

The 5A11 antigen forms homodimers. To determine if the 98 kDa species results from dimerization of the 5A11 antigen or association with a different polypeptide, cross-linked

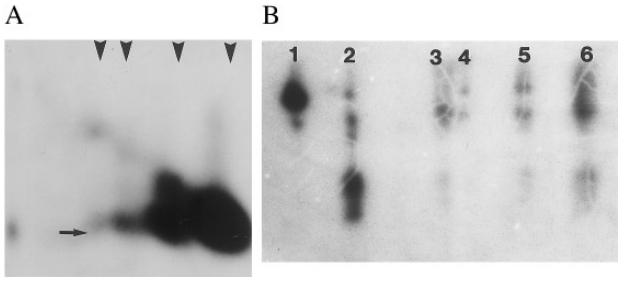

FIG. 2. Diagonal gel electrophoresis and proteolytic digestion of cross-linked 5A11 antigen. Immunoaffinity purified 5A11 antigen from ^{125}I-labeled erythrocytes treated with the thiol cleavable, membrane impermeable reagent DTSSP was subjected to two-dimensional gel electrophoresis (A). The locations of the four radiolabeled polypeptides of 50, 60, 80, and 98 kDa, after the first round of electrophoresis, are indicated (arrowheads). Reduction and separation in the second dimension produced species of 50 kDa in all samples (small arrow). (B) Proteolytic digestion with V8-protease of immunopurified 5A11 antigen. Lane 1, undigested 50-kDa antigen. Digestion of the 5A11 monomer (lane 2), 80 kDa (lanes 3 and 6), and 98 kDa (lanes 4 and 5) cross-linked species generated radiolabeled polypeptides with similar electrophoretic patterns.

species were immunoaffinity purified and separated by two-dimensional diagonal SDS-PAGE or subjected to partial proteolytic digestion. Electrophoresis in the first dimension under non-reducing conditions, separated 4 radiolabeled species, ranging from 50 to 98 kDa (Fig.2). Following diagonal gel SDS-PAGE under reducing conditions, the bulk of each migrated at a uniform rate, demonstrating the presence of radiolabeled polypeptides of similar electrophoretic mobility in the monomer and cross-linked species.

Limited proteolysis, with V8 protease, was used to determine if theses polypeptides represented homodimers of the 5A11 antigen or heterodimers. One would predict that if the cross-linking produced a heterodimer, the unique digestion pattern of the second polypeptide would be superimposed upon that of the 5A11 antigen, however, this was not observed. Similar cleavage patterns were obtained from the immunoaffinity purified 50 kDa (non-crosslinked), 80 kDa (cross-linked non-reduced) and 98 kDa (cross-linked reduced) polypeptides (Fig. 2) indicating the presence of only a single radiolabeled polypeptide in each sample.

The 98-kDa dimer from retina is part of a larger macromolecular complex. Triton X-100 solubilized proteins from retina were subjected to sedimentation velocity separation and Western blot analysis. Fast green staining of the blots revealed the distribution of total proteins in the gradient (Fig 3A). Immunolabeling revealed at least five distinct 5A11 species based upon sedimentation velocity and electrophoretic mobility (Fig 3B). Surprisingly, a significant portion of the 45.5 kDa polypeptide sedimented to fractions 18-19, the most dense fractions of the gradient. Sedimentation and electrophoretic separations performed on extracts prepared in the presence of BS3, demonstrated that the homodimer accounted for the immunoreactivity observed in fractions 18-19 (Fig. 3C).

The sedimentation rate of the 5A11 antigen dimer was compared by Western blot and immuno dot assay to proteins endogenous to the retina including the β-subunit of the Na+/K+-ATPase, NCAM, glutamine synthetase, and carbonic anhydrase II (13-17). Of these the most significant was to glutamine synthetase. Glutamine synthetase has a molecular mass of 43 kDa, and associates to form an holoenzyme with a mass $>$340 kDa, and a sedimentation value of S=15.2 (23). In our preparations, the majority of the glutamine synthetase immunoreactivity also localized to fractions 17-19. Direct comparison suggests association of the 5A11 antigen dimer with a large macro-molecular complex. In duplicate experiments, this association was not observed in erythrocyte preparations (data not shown) suggesting a unique interaction in the retina.

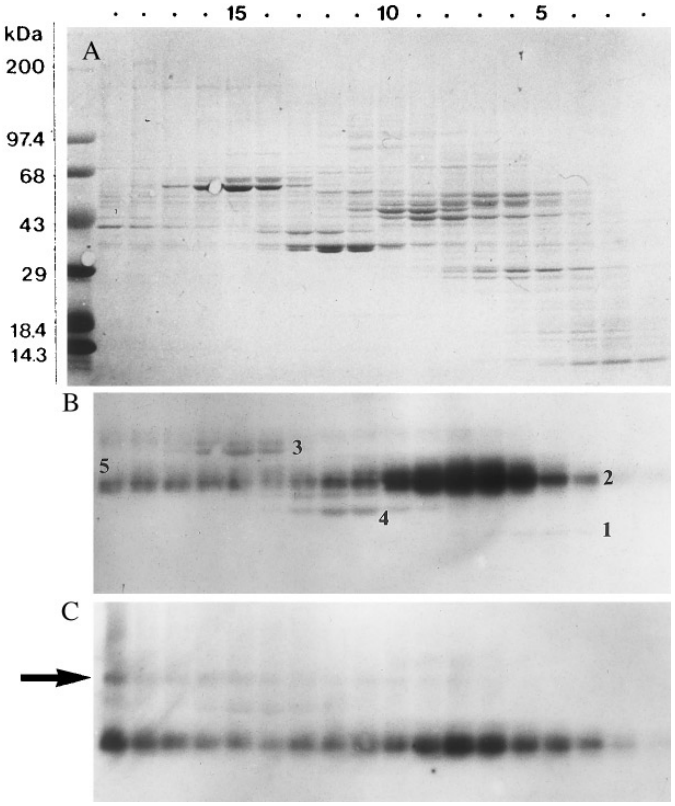

FIG. 3. Sedimentation velocity separation and immunoblot analysis of the 5A11 antigen. (A) Fast green staining of Triton X solubilized retina proteins fractionated by sedimentation through a sucrose gradient followed by Western blot analysis. Fraction 19 of the sucrose gradient corresponds to the bottom of the tube. (B) Probing of the blot with the 3B7 Mab resulted in immunolabeling of five discreet polypeptides: 1, nascent chain; 2, 45.5 kDa doublet in fractions 4-15; 3, 69 kDa in fractions 14-17; 4, 38 kDa; 5, 45.5 kDa in fractions 16-19. (C) Sedimentation velocity and immunoblot analysis of solubilized retina treated with 0.5 mM BS3. The cross-linked species were localized exclusively to the most dense fractions of the sucrose gradient (arrow). Locations of molecular weight markers are indicated.

DISCUSSION

In this report, several important features of the molecular interactions involving the 5A11 antigen are described: 1) The 5A11 antigen associates to form homo-oligomers in several tissues from chick; 2) Experiments on intact chick erythrocytes reveal that these oligomers represent the formation of a macromolecular complex on or within the plasma membrane of single cells, not the result of cell-to-cell interactions; 3) A fraction of the antigen in retina, namely the 98 kDa dimer, is a component of a large macromolecular complex as judged from the sedimentation rate in a sucrose gradient. This complex was not observed in erythrocyte preparations suggesting it did not result solely from association with the cytoskeleton or other membrane components (24). Rather, it is proposed that such occurs through association with a ligand on an opposing cell surface.

Based upon the current data, a hypothetical model of the 5A11 antigen can be deduced. We propose that the 5A11 antigen dimerizes to form a transmembrane receptor. Dimerization is functionally significant in the formation of numerous membrane receptors, including those of the immune system (25,26), growth factors (27), and the sea urchin egg receptor for sperm

(28). The proposed structure of the dimer would involve association of the transmembrane segments, pairing of the immunoglobulin like domains, and the possible association of the C-terminus with components of the cytoskeleton (24). The pairing of the immunoglobulin domains of the 5A11 dimer, reminiscent of immunoglobulin domains of antibodies, is the typical motif for paired structures of the IgSF (25) and is supported by the conservation in the human homologue of invariable amino acids present in most class II β-chains (29). Association with the cytoskeleton is suggested by the relative insolubility of the dimer to non-ionic detergent extraction, and corroborated by co-localization of the 5A11 antigen, spectrin and ankyrin to patches of the plasma membrane (24).

Prediction of a charged amino acid residue within the transmembrane domain of the 5A11/HT7 antigen and a leucine zipper motif spanning the transmembrane domain suggests formation of specific protein-protein interactions. Charged residues in or near the transmembrane domain are necessary for interaction of α and β chains of the T-cell receptor with polypeptides of the CD3 complex (26,30,31,32,33), and dimerization of class II MHC molecules (34). In an unrelated polypeptide, the cellular oncogene *neu*, a single base mutation resulting in a Val to Glu substitution within the membrane spanning domain results in receptor aggregation at the cell surface and a transformed cellular phenotype (35,36).

The leucine zipper motif (37,38,39) was first proposed in dimerization of DNA binding proteins. Sequence analyses of the 5A11/HT7 antigen predict a heptad repeat of leucine residues encompassing the membrane spanning domain, a single conserved substitution of phenylalanine for leucine at an additional site and a leucine at position-6 immediately preceding the heptad repeat (3,4,8,11). This alignment would also position the GLU^{197} to the opposite face of the α-helix relieving repulsive electrostatic interactions from pairing of like charged residues (37). The leucine zipper motif has been observed in or near the proposed transmembrane domains of numerous proteins (40,41,42), although some investigators suggest the use of caution when extending this interpretation to all cases (40,43).

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation Grants BNS-8819743 and Dir-8914602 and National Eye Institute Grant T32-EY07132. The β4B4 Mab was obtained from the developmental studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, and the Department of Biology, University of Iowa, under Contract NO1-HD-6-2915 from the NICHD.

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