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Abstract

Agrin is a basement membrane-associated proteoglycan that induces the formation of postsynaptic specializations at the neuromuscular junction. This activity is modulated by alternative splicing and is thought to be mediated by receptors expressed in muscle fibers. An isoform of agrin that does not induce postsynaptic specializations binds with high affinity to dystroglycan, a component of the dystrophin-glycoprotein complex. Transcripts encoding this agrin isoform are expressed in a variety of non-muscle tissues. Here, we analyzed the tissue distribution of agrin and dystroglycan on the protein level and determined their binding affinities. We found that agrin is most abundant in lung, kidney, and brain. Only a little agrin was detected in skeletal muscle, and no agrin was found in liver. Dystroglycan was highly expressed in all tissues examined except in liver. In a solid-phase radioligand binding assay, agrin bound to dystroglycan from lung, kidney, and skeletal muscle with a dissociation constant between 1.8 and 2.2 nM, while the affinity to brain-derived dystroglycan was 4.6 nM. In adult kidney and lung, agrin co-purified and co-immunoprecipitated with dystroglycan, and both molecules were co-localized in embryonic tissue. These data show that the agrin isoform expressed in non-muscle tissue is a high-affinity binding partner of dystroglycan and they suggest that this interaction, like that between laminin and dystroglycan, may be important for the mechanical integrity of the tissue.

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Agrin Is a High-affinity Binding Protein of Dystroglycan in Non-muscle Tissue*

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Matthias Gesemann‡§¶, Andrea Brancaccio¶‖, Beat Schumacher‡, and Markus A. Ruegg‡**

From the Departments of ‡Pharmacology and ‖Biophysical Chemistry, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

Agrin is a basement membrane-associated proteoglycan that induces the formation of postsynaptic specializations at the neuromuscular junction. This activity is modulated by alternative splicing and is thought to be mediated by receptors expressed in muscle fibers. An isoform of agrin that does not induce postsynaptic specializations binds with high affinity to dystroglycan, a component of the dystrophin-glycoprotein complex. Transcripts encoding this agrin isoform are expressed in a variety of non-muscle tissues. Here, we analyzed the tissue distribution of agrin and dystroglycan on the protein level and determined their binding affinities. We found that agrin is most abundant in lung, kidney, and brain. Only a little agrin was detected in skeletal muscle, and no agrin was found in liver. Dystroglycan was highly expressed in all tissues examined except in liver. In a solid-phase radioligand binding assay, agrin bound to dystroglycan from lung, kidney, and skeletal muscle with a dissociation constant between 1.8 and 2.2 nM, while the affinity to brain-derived dystroglycan was 4.6 nM. In adult kidney and lung, agrin co-purified and co-immunoprecipitated with dystroglycan, and both molecules were co-localized in embryonic tissue. These data show that the agrin isoform expressed in non-muscle tissue is a high-affinity binding partner of dystroglycan and they suggest that this interaction, like that between laminin and dystroglycan, may be important for the mechanical integrity of the tissue.

Agrin is a heparan sulfate proteoglycan that is required for the induction and regeneration of pre- and postsynaptic specializations at neuromuscular junctions (NMJ[1]; Refs. 1 and 2). When applied to cultured chick myotubes, agrin leads to the formation of protein aggregates containing acetylcholine receptors (AChRs), acetylcholinesterase, and several other molecules that are also found at the neuromuscular junction *in vivo* (3). The aggregating activity of agrin is strongly influenced by alternative mRNA splicing at two sites, called A and B in chick (y and z in rodents). While agrin isoforms that contain inserts at these sites are highly active in an AChR aggregation assay, an agrin isoform lacking the inserts, designated agrin$_{A0B0}$, is inactive (4–6). Agrin mRNA encoding isoforms with AChR-aggregating activity are expressed in motor neurons, while cells in non-nervous tissue synthesize agrin transcripts lacking inserts at sites A and B (agrin$_{A0B0}$; Refs. 4 and 7–12). Consequently, agrin-like immunoreactivity is detected in numerous non-neuronal tissues (13, 14).

In skeletal muscle and the electric organ of *Torpedo californica*, the major binding protein of agrin is α-dystroglycan (15–18). α-Dystroglycan originates from a precursor protein that becomes cleaved post-translationally giving rise to α-dystroglycan, a heavily glycosylated peripheral membrane protein that binds to laminin and agrin, and the transmembrane protein β-dystroglycan (19). Both molecules associate with several other proteins to form the dystrophin-glycoprotein complex (DGC; reviewed in Ref. 20). Mutations in genes that encode different members of the DGC cause several muscular dystrophies (reviews: 21, 22), suggesting that the DGC links the subsarcolemmal cytoskeleton and the muscle fiber's basement membrane to confer mechanical stability. Unlike some of the other components of the DGC, dystroglycan is expressed in a variety of tissues (19), and the recent observation that dystroglycan-deficient mice, which die early in development, fail to form a functional Reichert's membrane (23) suggests that dystroglycan is involved in organizing many basement membranes.

Laminin-1 and laminin-2 bind strongly to α-dystroglycan (24), suggesting that this interaction provides the main linkage of the DGC to basement membranes. Mutations in the gene coding for the α_2 subunit of laminin result in severe muscular dystrophies (25–27). Moreover, antibodies directed against laminin and α-dystroglycan perturb development of kidney (28, 29).

As a first step toward identifying possible functions of the non-AChR-aggregating agrin isoform agrin$_{A0B0}$, we have investigated its binding to α-dystroglycans isolated from different tissues. We find that agrin$_{A0B0}$ binds to α-dystroglycan isolated from adult chick lung, kidney, and skeletal muscle with a dissociation equilibrium constant of ~ 2 nM. Binding to α-dystroglycan from adult chick brain was approximately 2-fold lower. In addition, agrin co-localized with β-dystroglycan in developing chicken kidney and lung. In conclusion, these data provide evidence that agrin and α-dystroglycan bind strongly to each other outside of the NMJ, suggesting that agrin may have a similar function as the laminins in linking the basement membrane to the DGC.

EXPERIMENTAL PROCEDURES

Protein Purification, Labeling, and Immunoprecipitations—A 95-kDa COOH-terminal agrin fragment, c95$_{A0B0}$, expressed in COS-7 or 293 cells was purified by immunoaffinity column as described (6) using the monoclonal antibody 5B1 (13). Agrin fragments were iodinated as described elsewhere (30). Purification of α-dystroglycan was carried out

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§ Present address: The Salk Institute MNL-O, 10010 North Torrey Pines Rd., La Jolla, CA 92037.

¶ These authors contributed equally to this work.

** To whom correspondence should be addressed: Dept. of Pharmacology, Biozentrum, University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland. Tel.: 41-61-267-2246 or 41-61-267-2213; Fax: 41-61-267-2208; E-mail: rueegg@ubaclu.unibas.ch.

[1] The abbreviations used are: NMJ, neuromuscular junctions; AChR(s), acetylcholine receptors; DGC, dystrophin-glycoprotein complex; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

as described elsewhere (31) with the following modifications: about 15 g of freshly frozen tissue was homogenized in 40 ml of 50 mM Tris-HCl, pH 7.5 containing 0.2 M NaCl and 1 mM phenylmethylsulfonyl fluoride (buffer A) and incubated overnight with 10 ml of DEAE-Sephacel (Pharmacia Biotech Inc.). The resin was washed three times with 30 ml of buffer A, and bound proteins were eluted from the Sephacel with 20 ml of 50 mM Tris-HCl, pH 7.5, and 0.5 M NaCl (buffer B). The eluate was incubated with 2 ml of wheat germ agglutinin-Sepharose. After three washes with 20 ml of buffer B, elution from wheat germ agglutinin-Sepharose was achieved by adding 0.3 M *N*-acetylglucosamine. Eluted proteins were then dialyzed against 25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, pH 7.4 (TBS) and concentrated using Centricon™ filters (Amicon). For immunoprecipitations, 5 g of freshly frozen tissue was homogenized in 20 ml of buffer A containing 1% Triton X-100. After centrifugation at 39,000 × *g* for 15 min, the supernatant was passed through a filter paper. 750 μl of this filtrate was subjected to immunoprecipitations using 4 μl of anti-agrin antiserum (32). After incubation overnight at 4 °C, immunocomplexes were precipitated with protein A-Sepharose as described elsewhere (6). The presence of β-dystroglycan in the immunoprecipitates was analyzed by SDS-PAGE followed by Western blot analysis.

SDS-PAGE and Immunoblotting—Adult chicken tissues (about 1 g) were homogenized on ice with a 15-ml Dounce homogenizer in 10 ml of PBS containing a mixture of protease inhibitors (32). Homogenized tissues were subjected to SDS-PAGE on a 3–12% gradient gel. Proteins (~80 μg/lane) were transferred to nitrocellulose membranes as described elsewhere (33). In agrin blots the transfer was carried out in the absence of methanol. After blotting, membranes were washed once with water and then blocked with PBS containing 3% milk powder and 0.05% Tween 20 ($PBS^{M/T}$) for 2 h. Agrin immunoreactivity was detected by incubating blots with the anti-agrin antiserum raised against recombinant chick agrin (32) at a dilution of 1:2,000 in $PBS^{M/T}$. β-Dystroglycan was detected by the monoclonal antibody 8D5 (NovoCastra; Ref. 34), diluted 1:20 in $PBS^{M/T}$. Incubation time for both reactions was 90 min at room temperature. Nitrocellulose membranes were washed three times with $PBS^{M/T}$ and then subjected to a 45-min incubation with appropriate horseradish peroxidase-conjugated secondary antibodies at a dilution recommended by the manufacturer (Jackson ImmunoResearch Laboratories). After three washes with $PBS^{M/T}$ and one wash with PBS, immunoreactive protein bands were visualized by the ECL detection method (Amersham Corp.).

Transfer Overlay and Solid-phase Radioligand Binding Assays—Transfer overlay assays were done as described (30). Solid-phase radioligand binding assays were based on a protocol described in (30) with minor modifications. Briefly, α-dystroglycan was immobilized on microtiter plates by overnight incubation at 4 °C in 50 mM sodium bicarbonate, pH 9.6. Remaining binding sites were saturated by a 1-h incubation with TBS containing 1.25 mM $CaCl_2$, 1 mM $MgCl_2$ ($TBS^{+Ca/+Mg}$) and 3% BSA. After blocking, wells were incubated with $^{125}I\text{-}c95_{A0B0}$ at room temperature for 3 h in $TBS^{+Ca/+Mg}$ containing 3% BSA. The wells were washed four times with $TBS^{+Ca/+Mg}$, and bound radioactivity was counted with a γ counter. Each data point represents the average of triplicate wells ± S.D. Unspecific binding (binding in the presence of 2 μM unlabeled $c95_{A0B0}$) was subtracted from each data point. Curves were fitted by nonlinear regression analysis, assuming a single class of equivalent binding sites, using the equation counts/min = $(P \times c/(K_d + c))$, where counts/min represents radioactivity, K_d the binding dissociation constant, c the concentration of $^{125}I\text{-}c95_{A0B0}$, and P counts/min at saturation.

Immunofluorescence—Staining of frozen sections (12 μm) from embryonic chicken lung or kidney were done as follows. Tissues were cryoprotected by embedding them in PBS containing 20% sucrose before they were frozen in Tissue-Tek (Miles). Unfixed cryostat sections were blocked with PBS containing 3% BSA and 5% normal goat serum for 1 h and incubated with diluted (1:2,000) anti-agrin antibodies (32) for 1 h. After four washes with PBS, agrin immunoreactivity was visualized using Cy3-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) at a dilution of 1:1,000. β-Dystroglycan immunoreactivity in lung was detected by subsequent incubation of blocked slices with the monoclonal antibody 8D5 (NovoCastra; Ref. 34) followed by incubation with biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories) for 1 h. Conditioned medium from the hybridoma 8D5 was diluted 2-fold, whereas the biotinylated secondary antibodies were used at a dilution of 1:1,000. After four washes with PBS, sections were incubated with fluorescein isothiocyanate-conjugated streptavidin for 45 min and again washed four times with PBS. β-Dystroglycan in kidney slices was stained by a slightly different protocol. Sections were blocked with PBS containing 5% BSA, followed by a 1-h incubation with the monoclonal antibody 8D5. After four washes with PBS, goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) at a dilution of 1:1,000 were added for 45 min. β-Dystroglycan immunoreactivity was then visualized using Cy3-conjugated rabbit anti-goat IgG (1:1,000; Jackson ImmunoResearch Laboratories). After antibody incubation, slices were again washed, mounted on glass coverslips with Citifluor (City University), and analyzed under a Leica microscope equipped with epifluorescence optics.

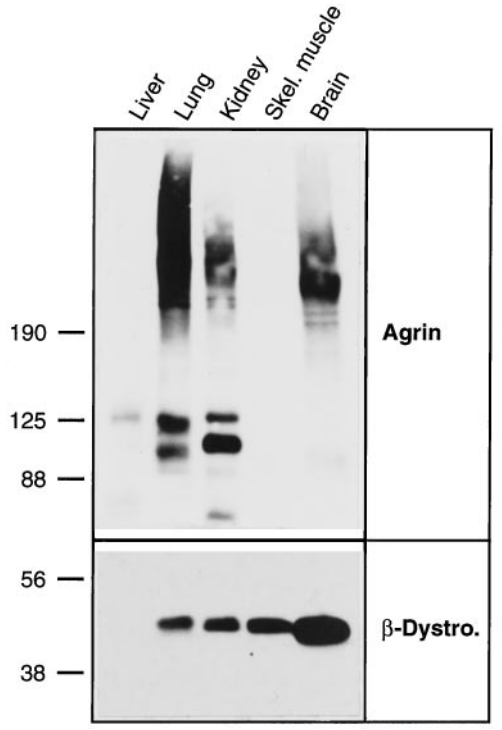

FIG. 1. **Agrin and dystroglycan are expressed in a variety of chick tissues.** Proteins were separated on a 3–12% SDS-polyacrylamide gradient gel and incubated with an anti-agrin antiserum (32) or the monoclonal anti-β-dystroglycan antibody 8D5 (34). In each lane, the same amount of protein was loaded. Agrin and β-dystroglycan are highly expressed in lung, kidney, and brain. Only a little agrin is found in skeletal muscle. In liver only some immunoreactive protein with a low M_r is detected. These immunoreactive bands with a lower M_r most likely represent proteolytic degradation products of agrin. β-Dystroglycan is highly expressed in skeletal muscle but is not detected in liver. No immunoreactive proteins were detected with the preimmune sera (not shown). Molecular masses of standard proteins in kilodaltons are given.

RESULTS

As a first step we analyzed the tissue distribution of agrin and one of its binding proteins, α-dystroglycan, in the adult chick. To this end, homogenates of brain, skeletal muscle, kidney, lung, and liver were separated by SDS-PAGE, blotted to nitrocellulose, and assayed for agrin and β-dystroglycan immunoreactivity by Western blot analysis. As shown in Fig. 1, agrin-like immunoreactivity with an apparent molecular mass between 400 and 600 kDa, was clearly detected in lung, kidney, and brain whereas no signal was seen in skeletal muscle and liver homogenates. Upon longer exposure agrin-like immunoreactivity with the high molecular mass could also be detected in skeletal muscle while we were not able to detect it in liver homogenates (data not shown). The tissue distribution of agrin in adult chicken brain, liver, and skeletal muscle is similar to that observed in the embryo, except that lower levels were detected in adult skeletal muscle (32). One explanation for this may be that in adult muscle, agrin expression is restricted to the NMJ (8, 13). Agrin-like immunoreactivity was also associated with discrete bands around 100 kDa. Proteins of similar size were purified from the electric organ of *T. californica* (35)

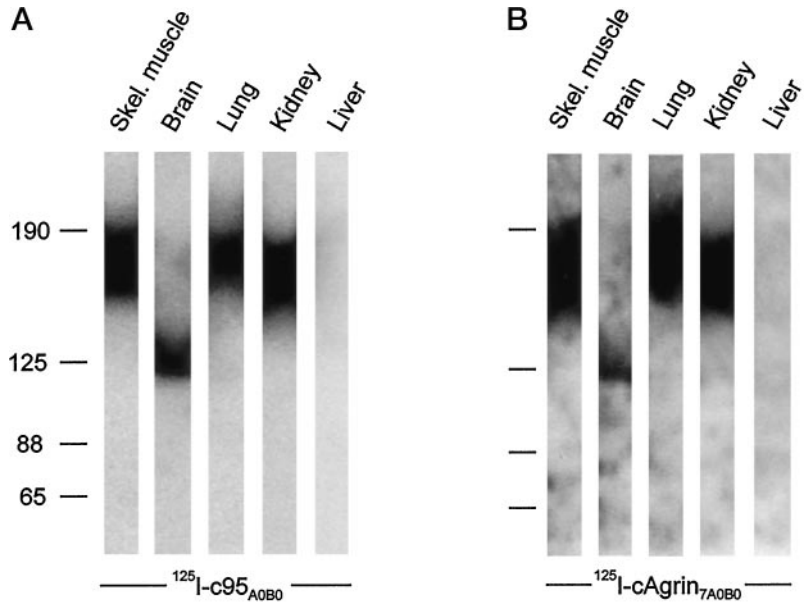

FIG. 2. **Transfer overlay assays with agrin$_{A0B0}$ to detect α-dystroglycans from different chick tissues.** Proteins were separated on a 6% SDS-PAGE, blotted to nitrocellulose, and overlaid either with a 95-kDa iodinated agrin fragment (^{125}I-c95$_{A0B0}$ (*A*)) or full-length chick agrin (^{125}I-cAgrin$_{7A0B0}$ (*B*)). Each lane was incubated with 2.5 nM iodinated agrin. Origin of α-dystroglycan preparation is indicated. Molecular masses of marker proteins in kilodaltons are shown.

and were also observed in embryonic chick and rat tissue (32, 36). These bands may represent proteolytic fragments derived from full-length agrin.

Since no antibodies are available that reliably recognize α-dystroglycan from chicken, we probed the same homogenates for the presence of β-dystroglycan, which is derived from the same precursor molecule and forms a tight complex with α-dystroglycan (19). As shown in Fig. 1, β-dystroglycan was most abundant in brain, followed by lung, kidney,and skeletal muscle, but β-dystroglycan was not detected in liver homogenates. Hence, the amount of β-dystroglycan in these tissues correlates with that of agrin except in skeletal muscle where β-dystroglycan was very abundant, but only a little agrin-like immunoreactivity was found.

Because lung, kidney,and muscle mainly express agrin transcripts encoding the splice variant lacking inserts at sites A and B (agrin$_{A0B0}$; Refs. 7, 8, 11, and 12), we next determined whether agrin$_{A0B0}$ binds to α-dystroglycan isolated from these tissues. To this end, binding of agrin$_{A0B0}$ to α-dystroglycan preparations isolated from a variety of tissues was analyzed by transfer overlay assays. As ligands, we used either iodinated full-length agrin (cAgrin$_{7A0B0}$) or the 95-kDa COOH-terminal fragment, c95$_{A0B0}$ (see Ref. 32 for nomenclature). As shown in Fig. 2, α-dystroglycan from all the tissues, except liver, gave a strong binding signal. No difference in α-dystroglycan binding was observed using recombinant full-length chick agrin (cAgrin$_{7A0B0}$) or its COOH-terminal half (c95$_{A0B0}$; Fig. 2), indicating that the NH_2-terminal part of agrin does not substantially contribute to the binding. While α-dystroglycan from lung, kidney, and skeletal muscle had an apparent molecular mass of 180 kDa, α-dystroglycan from brain was substantially smaller. Differences in the M_r of α-dystroglycan have been observed by others using laminin-1 as a ligand (31, 37, 38). Since the predicted molecular mass of the α-dystroglycan core protein is much smaller (~74 kDa; Ref. 19), the difference is most likely due to its extensive post-translational glycosylation (15, 38–40). The signals on transfer overlay assays were detected with as little as 2.5 nM iodinated ligand, suggesting a high-affinity binding of agrin to α-dystroglycan from all the tissues examined.

Since transfer overlay assays do not allow accurate measurement of binding affinity, we used radioligand binding assays to determine equilibrium constants for the binding of recombinant ^{125}I-c95$_{A0B0}$ to immobilized α-dystroglycan. As shown in Fig. 3, binding of c95$_{A0B0}$ to α-dystroglycan from skeletal muscle, lung, kidney, and brain was detected at low nanomolar concentrations. We measured K_d values between 2 and 5 nM (Table I), demonstrating that agrin$_{A0B0}$ binds to α-dystroglycan with high affinity. Binding of ^{125}I-c95$_{A0B0}$ to α-dystroglycan from all tissues was Ca^{2+}-dependent and was competed to more than 90% with 2 μM unlabeled c95$_{A0B0}$ (data not shown). The approximately 2-fold higher K_d for brain-derived α-dystroglycan is most likely due to its different glycosylation. In this context it is interesting to note that the binding of laminin-1 and laminin-2 to α-dystroglycan involves the carbohydrate moiety of α-dystroglycan (39–41).

Our data strongly suggest that agrin is a high-affinity binding partner for α-dystroglycan in kidney and lung. To see whether α-dystroglycan is associated with agrin *in situ*, we next tested the α-dystroglycan preparations from lung and kidney for the presence of agrin-like protein by Western blot analysis. As shown in Fig. 4*A*, agrin-like immunoreactivity was indeed detected in these preparations, but it had a reduced apparent molecular mass compared with agrin detected in tissue homogenates (see Fig. 1). As the binding site for dystroglycan is localized in the COOH-terminal part of agrin (see Fig. 2), the lower M_r of agrin found in α-dystroglycan preparations may be due to proteolytic cleavage within the amino-terminal portion of the molecule. Cleavage within this region would release a fragment that lacks the NtA domain of agrin, which is responsible for the binding of agrin to laminins (42). As the binding of agrin to laminin is of high affinity (42), proteolytic degradation may be requisite to extract agrin from tissue under the mild conditions used to purify α-dystroglycan. To assure that the presence of agrin-like immunoreactivity in the α-dystroglycan preparation reflected an association of both molecules *in situ*, we tested whether immunoprecipitation with anti-agrin antibodies from total tissue homogenates would also enrich for β-dystroglycan. As shown in Fig. 4*B*, β-dystroglycan was indeed present in these immunoprecipitations. These experiments thus show that agrin and α-dystroglycan are associated with each other *in situ* in adult kidney and lung.

To see whether agrin and dystroglycan could interact with each other during development, we next stained consecutive sections with anti-agrin or anti-β-dystroglycan antibodies. As shown in Fig. 5, agrin-like immunoreactivity was highly concentrated in basement membranes of lung bronchioles and kidney tubules. At the light microscopic level, β-dystroglycan

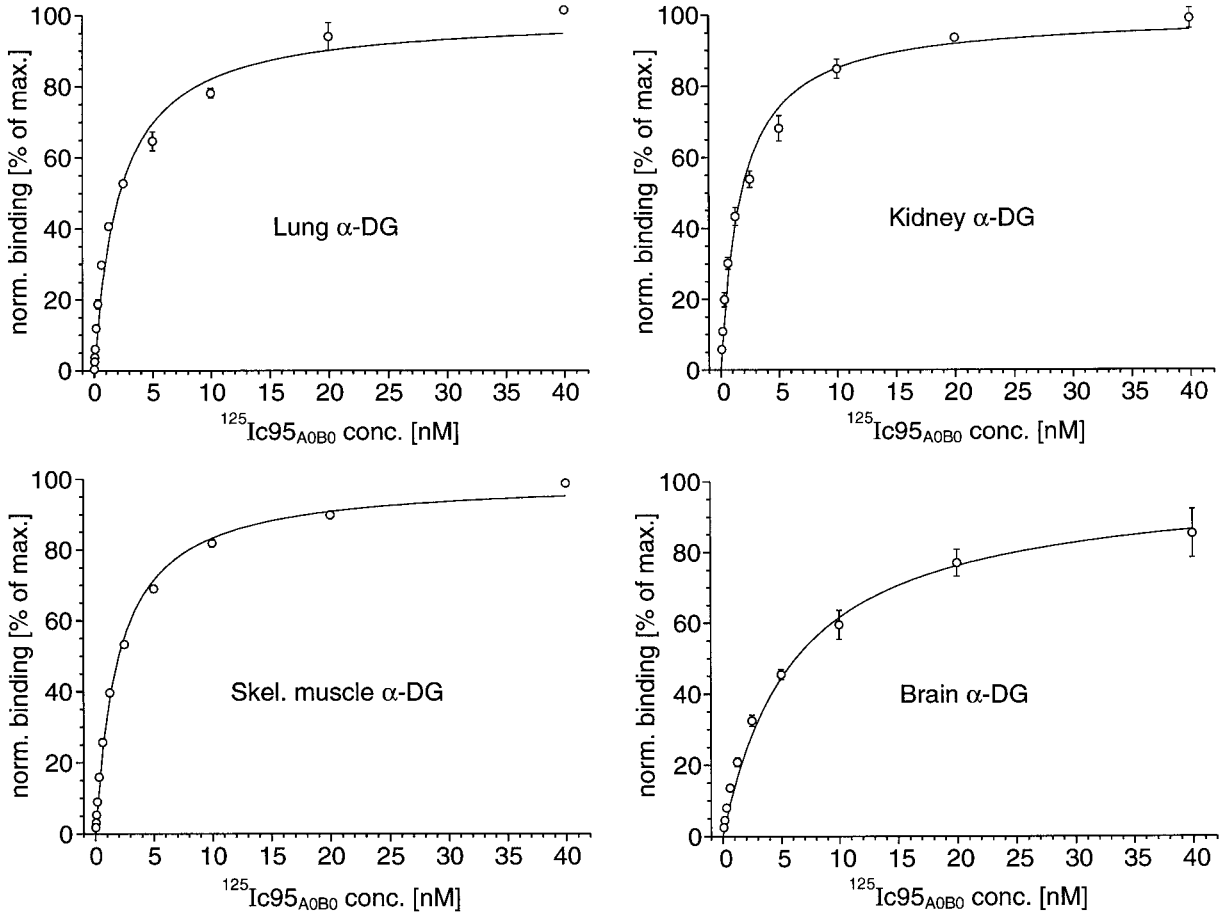

FIG. 3. **Solid-phase radioligand binding of agrin$_{A0B0}$ to α-dystroglycans.** Purified α-dystroglycan was coated to microtiter plates, blocked by 3% BSA, and incubated with iodinated agrin$_{A0B0}$. The tissue source of the α-dystroglycans is indicated. Curves were fitted by nonlinear regression analysis as described under "Experimental Procedures." To allow comparison of the binding curves from different tissues, data were normalized to the fitted value for radioactivity at total saturation. In addition, unspecific binding (*i.e.* binding in the presence of 2 μM unlabeled c95$_{A0B0}$) at each data point was subtracted. Data shown are the result of one representative experiment. Each value represents the mean ± S.D. of three measurements.

TABLE I
Dissociation binding constants of agrin to α-dystroglycans

Solid-phase radioligand binding was used to assess the affinity of agrin (c95$_{A0B0}$) to coated α-dystroglycan (see also Fig. 3). Experiments were performed at room temperature in TBS containing 1.25 mM $CaCl_2$ and 1 mM $MgCl_2$. At least three independent measurements were used to calculate individual dissociation constants.

α-Dystroglycan preparation (tissue)	K_d values ± S.E.
	nM
Lung	2.2 ± 0.8
Kidney	1.9 ± 0.7
Skeletal muscle	1.8 ± 0.3
Brain	4.6 ± 0.7

showed the same tissue distribution (Fig. 5, *right column*). No staining was detected with an agrin preimmune serum or in the absence of the β-dystroglycan antibody (data not shown). In summary, these data indicate that agrin and α-dystroglycan may also interact with each other during kidney and lung development.

DISCUSSION

Previous work on the binding of agrin and dystroglycan dealt mainly with the functional consequences of this interaction in the formation of the NMJ. It has been shown that the agrin isoform lacking inserts at sites A and B (agrin$_{A0B0}$), which is inactive in AChR aggregation, binds to skeletal muscle α-dystroglycan with higher affinity than the AChR-aggregating isoform agrin$_{A4B8}$ (18, 30, 43). Moreover, the region of agrin sufficient for AChR aggregation is distinct from the binding site for α-dystroglycan, making it unlikely that the binding of agrin to α-dystroglycan is involved in the signaling mechanisms initiated by agrin (30, 43).

In a solid-phase radioligand binding assay agrin$_{A0B0}$ binds to skeletal muscle α-dystroglycan with a dissociation constant of less than 2 nM (Table I). Using the same binding assay, a K_d value of 8 nM was determined for laminin-1 and a mixture of laminin-2 and laminin-4 (24). Hence, agrin seems to bind to muscle α-dystroglycan with higher affinity than the laminins. We also noticed this difference in the binding affinities in inhibition experiments. While an excess of unlabeled agrin$_{A0B0}$ was always very efficient in inhibiting the binding of iodinated laminin-1 to α-dystroglycan, the same concentration of unlabeled laminin-1 inhibited the binding of iodinated agrin$_{A0B0}$ only partially.[2]

In mature muscle fibers, agrin is concentrated at the NMJ and only little is detected in extrasynaptic regions (Fig. 1; Refs. 8 and 13). Agrin is therefore unlikely to be the major binding partner of α-dystroglycan in muscle fibers outside of the NMJ; instead, laminin-2 may have this role. The situation may, however, be different in adult kidney and lung, where agrin is expressed at high levels (Fig. 1). Lung and kidney, like muscle fibers, express transcripts encoding agrin$_{A0B0}$ (11, 12). Our

[2] M. Gesemann and M. A. Ruegg, unpublished observation.

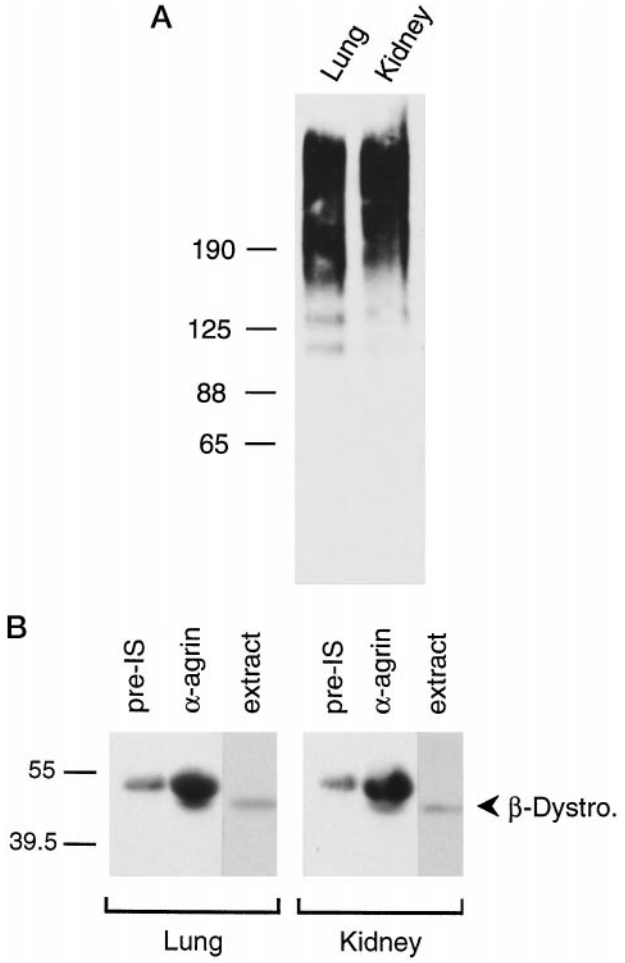

FIG. 4. **Agrin is complexed with α-dystroglycan *in situ*.** *A*, Western blot analysis using anti-agrin antibodies on α-dystroglycan purified from lung and kidney. No immunoreactive proteins were detected with an agrin preimmune serum (not shown). *B*, β-dystroglycan co-immunoprecipitates with agrin from adult lung and kidney. Western blot analysis using anti-β-dystroglycan antibodies on tissue extracts that had been precipitated with preimmune serum (*pre-IS*) or anti-agrin antiserum (*α-agrin*). As a control, total extract (*extract*) was included. In lung and kidney, β-dystroglycan is enriched in the agrin immunoprecipitates, while no specific signal is seen using preimmune serum. The band with a slightly higher molecular mass than β-dystroglycan, also seen with preimmune serum, represents cross-reactivity of the secondary antibody with the heavy chain of rabbit IgG. The blots using tissue extracts were exposed seven times longer than serum-treated samples. SDS-PAGE in all cases was 3–12% polyacrylamide gradient gels. Molecular masses in kilodaltons of standard proteins are given.

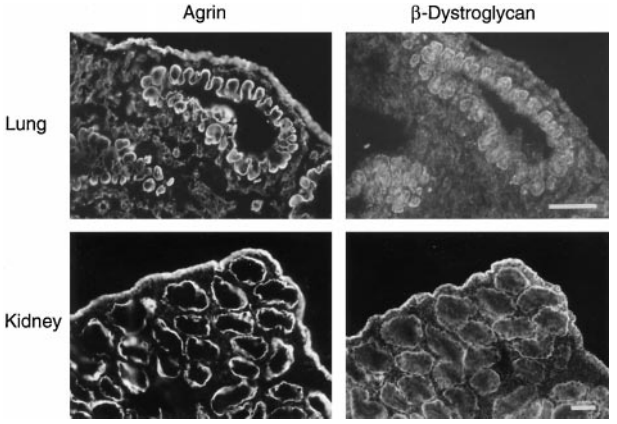

FIG. 5. **Immunolocalization of agrin and β-dystroglycan during development of chick lung and kidney.** Consecutive frozen sections of 15-day-old embryonic kidney and lung were stained either with anti-agrin or anti-β-dystroglycan antibodies as indicated. Both agrin and β-dystroglycan are localized in the basement membranes of developing kidney tubules or lung bronchioles. *Bars* = 50 μm.

study now shows that $agrin_{A0B0}$ binds to α-dystroglycan from both tissues with high affinity (K_d values ~2 nM) and that agrin is complexed with dystroglycan *in vivo* (Figs. 4 and 5). Hence, the major binding protein for α-dystroglycan in adult kidney and lung may be agrin and not the laminins.

In kidney, the binding of laminin-1 to α-dystroglycan has been suggested to be important during epithelial morphogenesis. This is mainly based on perturbation studies using polyclonal antibodies directed against proteolytic fragments E8 or E3 of laminin-1 (28) or the function-blocking monoclonal antibody IIH6 directed against α-dystroglycan (29). In those studies, the antibodies perturbed the differentiation of mesenchyme into epithelium in organ cultures of embryonic kidneys. Here we show co-localization of β-dystroglycan and agrin in developing chick kidney tubules and lung bronchioles (Fig. 5). Although we used anti-β-dystroglycan antibodies instead of anti-α-dystroglycan antibodies, it is likely that the tissue distribution of the two proteins does not differ. Both proteins originate from the same precursor molecule (19), they tightly associate with each other, and in other species, no differences in the tissue distribution of α- and β-dystroglycan have been observed. We therefore conclude that agrin is highly expressed in developing chick kidney and lung and that it co-distributes with α-dystroglycan. This co-localization hence suggests that binding of agrin to α-dystroglycan may also be possible during embryogenesis. Although we have not looked at the tissue distribution of agrin and α-dystroglycan at early stages of kidney and lung development, we hypothesize that part of the perturbation of kidney development with the monoclonal antibody IIH6 (29) might be due to inhibition of the agrin-dystroglycan interaction. Hence, it would be interesting to study the effect of anti-agrin antibodies on the development of kidney.

We have also determined the binding affinity of $agrin_{A0B0}$ to α-dystroglycan isolated from adult brain. We find that this binding is of more than 2-fold lower affinity than that to α-dystroglycan from skeletal muscle, kidney, and lung (Table I). The weaker binding of agrin is paralleled by a decrease in the apparent M_r of brain α-dystroglycan, suggesting an involvement of carbohydrates in the binding (37, 44). Consistent with this, glycosylation of α-dystroglycan is critically involved in the binding of laminin-1 (39–41). Again, the functional significance of the binding of agrin to brain-derived α-dystroglycan is not yet clear. Agrin-like immunoreactivity in the brain is highly concentrated in the basal lamina of certain microvessels that serve as selective filtration barriers. The endothelial cells lining the blood capillaries express the splice variant $agrin_{A0B0}$, which binds most strongly to α-dystroglycan (12). Blood capillaries in the nervous system also express α-dystroglycan, utrophin, and α_2 chain-containing laminins (45–47). The presence of agrin and dystroglycan in blood capillaries and the finding that agrin expression is high when a functional blood-brain barrier is established (48) suggest a role of the dystroglycan-agrin binding in blood-brain barrier formation. Alternatively, the binding of agrin to α-dystroglycan may play a role in the maintenance of synaptic structures as DGC-like immunoreactivity is detected at synapses in the central nervous system (47, 49). Similarly, agrin-like immunoreactivity has been found at synapses in the chick retina (50).

No gross abnormalities in the tissues examined here have been reported in the agrin-deficient mice (2). In these knockout mice, only the exons encoding site B were removed. As a consequence, even in homozygous knockout animals, agrin isoforms lacking the B-site were still expressed, although at a

much lower level. As the binding affinity of agrin to α-dystroglycan is high, the low level of agrin may have been sufficient for a functional interaction with α-dystroglycan. Animals that are completely deficient in agrin may be required to reveal the functional consequences of the loss of the binding of agrin to α-dystroglycan.

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