We determined the specificity of two hamster monoclonal antibodies and a sheep polyclonal antiserum against heparan sulfate proteoglycan isolated from rat glomerular basement membrane. The antibodies were characterized by enzyme-linked immunosorbent assay on various basement membrane components and immunoprecipitation with heparan sulfate proteoglycan with or without heparitinase pre-treatment. These experiments showed that the antibodies specifically recognize approximately 150-, 105-, and 70-kDa core proteins of rat glomerular basement membrane heparan sulfate proteoglycan. Recently, we showed that agrin is a major heparan sulfate proteoglycan in the glomerular basement membrane (Groffen, A. J. A., Ruegg, M. A., Dijkman, H. B. P. M., Van der Velden, T. J., Buskens, C. A., van den Born, J., Assmann, K. J. M., Monnens, L. A. H., Veerkamp, J. H., and van den Heuvel, L. P. W. J. (1998) J. Histochem. Cytochem. 46, 19–27). Therefore, we tested whether our antibodies recognize agrin. To this end, we evaluated staining of Chinese hamster ovary cells transfected with constructs encoding full-length or the C-terminal half of rat agrin by analysis on a fluorescence-activated cell sorter. Both hamster monoclonals and the sheep antiserum clearly stained cells transfected with the construct encoding full-length agrin, whereas wild type cells and cells transfected with the construct encoding the C-terminal part of agrin were not recognized. A panel of previously characterized monoclonals, directed against C-terminal agrin, clearly stained cells transfected with either of the constructs but not wild type cells. This indicates that both hamster monoclonals and the sheep antiserum recognize epitopes on the N-terminal half of agrin. By immunohistochemistry on rat renal tissue, we compared distribution of N-terminal agrin with that of C-terminal agrin. The monoclonal antibodies against C-terminal agrin stained almost exclusively the glomerular basement membrane, whereas the anti-N-terminal agrin antibodies recognized all renal basement membranes, including tubular basement membranes. Based on these results, we hypothesize that full-length agrin is predominantly expressed in the glomerular basement membrane, whereas in most other renal basement membranes a truncated isoform of agrin is predominantly found that misses (part of) the C terminus, which might be due to alternative splicing and/or posttranslational processing. The possible significance of this finding is discussed.

Heparan sulfate proteoglycans (HSPGs) are composed of a core protein to which anionic heparan sulfate (HS) side chains are attached. Apart from its presence in basement membranes (BMs), HSPG is found in other extracellular matrices and on many, if not all, cell surfaces (1–3). The first extracellular matrix HSPG that was isolated (from mouse Engelbreth-Holm-Swarm sarcoma), characterized, and cloned was perlecan, named after its beads-on-a-string-like appearance in rotary shadowing electron microscopy (4–6). Different isoforms of perlecan have been found, which appear to be the result of alternative splicing (7, 8). Agrin is another extracellular matrix HSPG that is not related to perlecan (9, 10). Agrin was first isolated from the electric organ of Torpedo (11) and was recently identified as an HSPG (12). During formation of the neuromuscular junction, motor neurons provide a number of signals that promote the differentiation of the post-synaptic membrane. An early signal is the secretion of specific isoforms of agrin and their incorporation into the basal lamina. Agrin initiates the clustering of nicotinic acetylcholine receptors and other components of the muscle cell surface at the site of contact with axons of motor neurons (13–16). The structure of the agrin molecule and the functions of the various domains is extensively studied and presently well understood (17). The C terminus of agrin contains several homology regions with the G-domain of laminin and four epidermal growth factor-like repeats (18). The binding site of agrin to α-dystroglycan is located on the C terminus as well. Agrin binds to α-dystroglycan via its laminin G repeats (19). Furthermore, a heparin/HS binding site is located on the C terminus and binding of heparin or HS prevents clustering of the acetylcholine receptors, indicating that HSPGs mediate this activity (20–22). Most alternative splicing sites have been identified in the C terminus, three on rat agrin called x, y, and z and two on chick and Torpedo agrin called A and B, corresponding to rat y and z. This alternative splicing affects the activity of agrin to cluster acetylcholine receptors. The most active forms only occur in neural...
tissue, whereas the isoforms from non-neural tissue are less active (14, 23–25). This suggests that splicing is regulated in a tissue-specific manner. The N-terminus of agrin contains nine domains homologous to the Kazal family of protease inhibitors and several laminin III homology regions (18). On the N-terminal half of agrin, several attachment sites for HS side chains are found. Up until now, only one alternative splicing site has been found in the N-terminus. The insert on the N-terminal half is required for binding to the extracellular matrix which is mediated by laminin (26–28). Expression of agrin is not restricted to neurons. Agrin mRNA can be detected in a wide range of different tissues (24), and the agrin protein has been demonstrated to be a basal lamina component in many tissues including kidney during early development (29). Recently, we have shown the presence of agrin in the glomerular basement membrane (glomerular BM) in adult human kidney (30).

In this study, we raised and characterized two hamster monoclonal antibodies (mAbs) and a sheep polyclonal antiserum against rat glomerular BM HSPG. The specificity of the mAbs is studied on Chinese hamster ovary (CHO) cells that are transfected with constructs coding for full-length agrin or the C-terminal half of agrin. These studies showed that these antibodies recognize epitopes on the N-terminal half of agrin. The staining on rat kidney tissue of these mAbs is compared with that of different mAbs against well-defined epitopes on the C terminus of agrin. These experiments suggest that most renal BMs except the glomerular BM express a truncated isoform of agrin that lacks (part of) the C-terminal half due to alternative splicing and/or posttranslational processing. We speculate on the possible involvement of the C terminus of agrin in glomerular function.

MATERIALS AND METHODS

**HSPG Purification**

For immunization, rat glomerular HSPG was isolated from rat glomeruli (31). Proteoglycans were isolated by guanidine extraction, dialysis against urea, DEAE ion-exchange chromatography, chondroitinase ABC digestion (Sigma) and by fast protein liquid Q-Sepharose chromatography. For immunoprecipitation, HSPG was isolated from rat glomerular BM obtained by a detergent procedure (32), from which proteoglycans were isolated as described (31). All procedures were performed at 4 °C in the presence of 10 mM sodium EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine HCl, and 10 mM N-ethylmaleimide.

**Antibody Production**

Male Chinese hamsters were immunized with rat glomerular HSPG according to a previously described protocol (31). Hybridomas were screened in indirect immunofluorescence (IF) on rat kidney cryostat sections (see below) for a staining pattern comparable with the staining of previously described mAbs against human glomerular BM HSPG, which primarily stain the glomerular BM in a bright linear pattern and the tubular BMs to a lesser extent (32). Two hybridomas were found to meet these criteria, called MI-90 and MI-91, and were subcloned twice by limiting dilution so that all subclones displayed the same reactivity pattern. From these two clones, ascites production was elicited in nude mice.

A sheep was immunized intracutaneously with rat glomerular HSPG according to a previously described protocol (32), and after the last booster the sheep was bled and the serum (called GR-14) was collected.

**Characterization of Antibodies**

**Enzyme-linked Immunosorbent Assay**—The reactivity of the antibodies with various BM components was determined in ELISA as described (34). Rat glomerular BM HSPG, human glomerular BM HSPG (isolated as described, Ref. 32), rat L2-laminin, mouse fibronectin, mouse collagen type IV (all from Sigma; all at 1 μg/well), and HS from bovine kidney (Seikagaku Kogyo, Tokyo, Japan; 5 μg/well) were coated in PBS. Primary antibodies MI-90 and MI-91 were diluted 1:1,000, and GR-14 was diluted 1:1,000. The following peroxidase-conjugated secondary antibodies in a dilution of 1:500 were used: rabbit anti-hamster IgG (Nordic, Tilburg, The Netherlands) for mAb MI-90, goat anti-hamster IgG (Cappel-Organon Teknika, Turnhout, Belgium) for mAb MI-91, and rabbit anti-sheep IgG (Cappel) for GR-14.

**Immunoprecipitation and SDS-PAGE**—Rat glomerular BM HSPG was labeled with 125I according to the chloramine T-method (35). Part of the sample was treated with heparitinase (EC 4.2.2.8; from Sigma) 1 unit/ml in 10 mM Hepes, pH 7.0, containing 2 mM CaCl₂ for 6 h at 43 °C. Radiolabeled HSPG was pre-absorbed with protein G-Sepharose CL-4B to remove eventual radiolabeled IgG. Normal hamster IgG and culture supernatants of mAbs MI-90 and MI-91 were incubated with protein G-Sepharose, to which goat anti-hamster IgG (Cappel) was immobilized. Normal mouse IgM (MOPC-104E; Bionetics, Kensington, MD) and mouse anti-HS side chain mAb JM-403 (31, 36) were incubated with protein G-Sepharose to which goat anti-mouse IgM (Cappel) was immobilized. Normal sheep IgG and sheep antiserum GR-14 were directly coupled to protein G-Sepharose. After washing with PBS-Tween, the immobilized antibodies were incubated overnight at 4 °C with radiolabeled rat glomerular BM HSPG (with or without heparitinase pretreatment). After washing in PBS-Tween, sample buffer containing 5% β-mercaptoethanol was added, the sample was boiled for 3 min and centrifuged, and the supernatant was subjected to polyacrylamide gel electrophoresis on a 7% homogenous gel. The gel was dried and used for autoradiography for 3 days at ~70 °C.

**Agrin-transfected CHO Cells**—To investigate the reactivity of our antibodies with agrin, we used CHO cells, stably transfected with constructs encoding full-length rat agrin or the C-terminal half of agrin (construct N1, amino acids 1137–1940) (17). The cells were cultured as described (17) and continuously selected for the presence of the construct by Geneticin (G418, 500 μg/ml; Life Technologies, Inc., Paisley, Scotland). Untransfected CHO cells were used as a negative control. On cytospins of CHO cells, indirect IF was performed with anti-HSPG mAbs MI-90, MI-91 antiserum GR-14, and anti-agrin mAb Agr-131 (17) (Table I) as described for the kidney cryostat sections (see below). For analysis by fluorescence-activated cell sorter (FACS), samples of 100 μl of cell suspension containing 5 × 10⁵ cells were permeabilized and fixed with 1 ml of PermeaFix (Ortho Diagnostic, Beere, The Netherlands) according to the instructions of the manufacturer. The cells were incubated with 100 μl of primary antibody (Table I) for 30 min at 0 °C. The cells were washed and incubated with fluorescein isothiocyanate isothiocyanate-conjugated secondary antibody for 30 min at 0 °C. The cells were washed twice and kept in 1% paraformaldehyde until analysis on a Coulter Epics XL flow cytometer (Coulter Corp., Hialeah, FL). Mean fluorescence was determined on a scale between 0 and 1024, and values were corrected for staining by the secondary antibody alone.

**Indirect Immunofluorescence**

Indirect IF was performed as described previously (34) on 2 μm cryostat sections of rat kidney. To evaluate the specificity of the hamster and sheep antibodies for the HSPG core protein, pretreatment of rat kidney cryostat sections was performed by incubating nonfixed sections with 0.25 units/ml heparitinase in 10 mM Hepes, pH 7.0, containing 2 mM CaCl₂ for 30 min at 37 °C.

**RESULTS**

**Antibody Reactivity with Basement Membrane Components in ELISA**—The reactivity of hamster mAbs MI-90 and MI-91

### Table I

| Primary antibody | Antigen | Dilution | Secondary antibody | Dilution | Source |
|-----------------|---------|----------|--------------------|----------|--------|
| MI-90           | Rat glom HSPG | 1:400    | Goat anti-hamster IgG | 1:50     | Jackson, West Grove, PA |
| MI-91           | Rat glom HSPG | 1:800    | Goat anti-hamster IgG | 1:200    | Cappel-Organon Teknika, Turnhout, Belgium |
| GR-14           | Rat glom HSPG | 1:800    | Rabbit anti-sheep IgG | 1:100    | Southern, Birmingham, AL |
| Agr-30, −33, −131 | Rat agrin | 1:1000   | Sheep anti-mouse IgG F(ab)₂ | 1:500 | Organon Teknika, Turnhout, Belgium |
| −137, −435     |         |          |                    |          |        |
and sheep antiserum GR-14 in ELISA with different BM components is shown in Fig. 1. For MI-90, a clear reactivity was observed with both rat glomerular BM HSPG and human glomerular BM HSPG. For MI-91, a reactivity was observed with rat glomerular BM HSPG, but not with human glomerular BM HSPG. Neither of the mAbs bound to rat L2-laminin, mouse fibronectin, mouse collagen type IV, or HS. Antiserum GR-14 bound to rat glomerular BM HSPG, human glomerular BM HSPG, and to a lesser extent to rat L2-laminin, but not to the other BM components.

**Immunoprecipitation and SDS-PAGE**—After immunoprecipitation of intact, 125I-labeled rat glomerular BM HSPG with hamster mAbs MI-90, MI-91, and sheep antiserum GR-14, gel electrophoresis and autoradiography revealed a broad smear with a molecular mass larger than 220 kDa, which might have resulted from different core proteins and/or different extent of glycosylation (Fig. 2, lanes 2, 3, and 7). This is in agreement with previous findings (32). The same band is seen with the mouse anti-HS side chain mAb JM-403 (lane 5) (33). To assess the molecular mass of the HSPG core protein(s), HSPG was treated before immunoprecipitation with heparitinase to degrade the HS glycosaminoglycan side chains. This resulted in a major band of ~170 kDa and several bands with molecular masses of ~105 and 70 kDa, which were equal for MI-90, MI-91, and GR-14 (lanes 9, 10, and 14). mAb JM-403 directed against HS side chain, however, is negative after treatment with heparitinase, as expected (lane 12). Non-relevant hamster IgG (lanes 1 and 8), mouse IgM (lanes 4 and 11), and normal sheep IgG (lanes 6 and 13) with the same Ig concentration served as negative controls. These results indicate that the mAbs MI-90 and MI-91, and the sheep antiserum GR-14 are directed against the core protein(s) of an HSPG present in rat glomerular BM.

**Indirect Immunofluorescence on CHO Cells Transfected with Agrin Constructs**—To evaluate recognition of rat agrin by our antibodies, indirect IF was performed on CHO cells transfected with constructs encoding full-length agrin (37) or the C-terminal half of agrin (N1) (25). Binding of antibodies was evaluated either microscopically on cytopsins or by FACS analysis. First, microscopic evaluation of cytopsins showed that both hamster anti-HSPG mAbs MI-90 and MI-91 and sheep anti-HSPG GR-14 recognized full-length agrin as expressed by CHO cells transfected with full-length agrin (Fig. 3, A-C), whereas they did not bind to the C-terminal half of agrin as expressed by cells transfected with construct N1 (Fig. 3, E-G, Table II). Neither did they bind to wild type CHO cells. The positive control anti-agrin mAb Agr-131 bound to both full-length agrin and to the C-terminal half of agrin as expressed by cells transfected with full-length agrin or construct N1, respectively (Fig. 3, D and H, Table II) To confirm this crucial conclusion, we also performed fluorescence analysis by FACS and quantified mean fluorescence intensity. As shown in Table II, full-length agrin expressed by CHO cells is recognized by both hamster mAbs and the sheep antiserum. These antibodies, however, were negative on N1-transfected cells and on wild type CHO cells. Anti-agrin mAb Agr-131, however, recognized both full-length and C-terminal agrin. These results indicate that hamster mAbs MI-90 and MI-91 and the sheep antiserum GR-14 recognize epitopes on the N-terminal half of agrin.

**Distribution of Agrin in Rat Kidney**—In indirect IF, mAbs MI-90 and MI-91 stain most BMs in a linear way, the glomerular BM with the highest intensity (Fig. 4, A and B). This staining pattern closely resembles the staining of previously developed mAbs directed against human glomerular BM HSPG (38). Only mAb MI-91 weakly stained Bowman’s capsule and BMs of smooth muscle cells in arteries and arterioles. Both mAbs stained BMs of endothelial cells in blood vessels but not the mesangial matrix and peritubular capillaries. (Fig. 4, A and B) mAb MI-90 also stained human kidney cryostat sections, and mAb MI-91 also stained mouse kidney cryostat sections. (Table III). The polyclonal antiserum GR-14 stained the glo-
merular BM, all tubular BMs, and BMs of endothelial cells in a bright linear way but not the mesangial matrix nor the peritubular capillaries. Bowman’s capsule, and the smooth muscle cells in arteries and arterioles were stained weakly (Fig. 4C). This staining pattern is similar to that of a previously described polyclonal antibody directed to human glomerular BM HSPG (32). GR-14 also stained human and mouse kidney cryostat sections. (Table III) The staining pattern of MI-90, MI-91 and GR-14 could not be prevented by pre-treatment of the sections with heparitinase, indicating that the antibodies are directed against the core protein of HSPG. mAb JM-403 directed against the HS side chain, however, was negative after treatment with heparitinase, as expected. This confirms our data from the immunoprecipitation with heparitinase-pre-treated glomerular BM HSPG (Fig. 2, lanes 8–14).

Five different mAbs directed against well-defined domains in the C-terminal half of agrin were described earlier (17). They predominantly stain the glomerular BM, whereas most other BMs were not stained at all. Also, Bowman’s capsule is stained weakly and segmentally (Fig. 4D, Table III). This staining is clearly different from that of our hamster mAbs which are directed against N-terminal domains of agrin and stain almost all renal BMs. These data suggest that different isoforms of agrin are expressed in the kidney. A large agrin isoform is found in the glomerular BM, containing both the C-terminal part (stained by five mouse anti-C-terminal agrin mAbs) and N
terminus (stained by both hamster mAbs and the polyclonal antiserum). On the contrary, one or more smaller isoforms are found in all other renal BMs, which miss (part of) the C terminus (negative with all mAbs against the C-terminal half of agrin), but contain the N terminus of agrin (stained by our hamster mAbs and the sheep antiserum). This conclusion could also explain our results in the immunoprecipitation study in which several core proteins, differing with respect to their molecular mass, were found by the hamster and sheep antibodies. However, these differences in molecular size can also be due to proteolysis.

**DISCUSSION**

In the present study, we characterized the N-terminal agrin specificity of two hamster mAbs MI-90 and MI-91 and a sheep polyclonal antiserum GR-14 raised against rat glomerular BM HSPG. Furthermore, we showed a differential agrin expression in renal BMs by comparing our antibodies with well-defined mAbs directed against the C terminus of agrin. The specificity of the antibodies was evaluated by means of ELISA on various BM components and by immunoprecipitation with isolated rat glomerular BM HSPG. In these experiments, it is shown that

**TABLE II**

*Cytospins or FACS of CHO cells transfected with constructs coding for either full-length agrin or the C-terminal half of agrin or wild type CHO cells stained with hamster mAbs MI-90, MI-91, sheep antiserum GR-14, or anti-agrin mAb Agr-131. Staining on cytospin preparations is quantified as follows: –, negative; +, weak; ++, moderate; +++, strong. Staining intensity on the FACS is quantified as mean fluorescence intensity (on a scale from 0 to 1024). Results from one representative out of four experiments are shown.*

| Antibody | Wild type Cytospin | FACS | Full-length agrin Cytospin | FACS | C-terminal half agrin Cytospin | FACS |
|----------|-------------------|------|----------------------------|------|-------------------------------|------|
| MI-90    | –                 | 0    | +++                        | 306  | –                             | 0    |
| MI-91    | –                 | 7    | +++                        | 412  | –                             | 0    |
| GR-14    | ±                 | 54   | ++                         | 178  | ±                             | 63   |
| Agr-131  | ±                 | 51   | +++                        | 326  | ++                            | 163  |

**FIG. 4**  
Agrin staining of MI-90, MI-91, GR-14, or Agr-131 on rat kidney. Indirect immunofluorescence with MI-90 (A), MI-91 (B), GR-14 (C), anti-agrin Agr-131 (D) on rat kidney cryostat sections (magnification, × 200; exposure time, 8 s).
Molecular masses of protein(s) of an HSPG present in rat glomerular BM with both hamster mAbs and the sheep antiserum recognize the core muscle; ND, not determined.

TABLE III

| Antibody | Rat | Human | Mouse |
|----------|-----|-------|-------|
| MI-90    | ++  | +     | -     |
| MI-91    | ++  | +     | +     |
| GR-14    | +   | -     | +     |
| Agr-131  | ++  | -     | +     |

Kidney staining of agrin by MI-90, MI-91, GR-14, and Agr-131 on rat, human, and mouse renal tissue. GBM, glomerular basement membrane; TBM, tubular basement membrane; MM, mesangial matrix; BC, Bowman's capsule; PTC, peritubular capillaries; VSM, vascular smooth muscle; ND, not determined.

Direct immunofluorescence of MI-90, MI-91, GR-14, or Agr-131 on rat, human, and mouse renal tissue. GBM, glomerular basement membrane; TBM, tubular basement membrane; MM, mesangial matrix; BC, Bowman's capsule; PTC, peritubular capillaries; VSM, vascular smooth muscle; ND, not determined.

Indirect immunofluorescence of MI-90, MI-91, GR-14, or Agr-131 on rat, human, and mouse renal tissue. GBM, glomerular basement membrane; TBM, tubular basement membrane; MM, mesangial matrix; BC, Bowman's capsule; PTC, peritubular capillaries; VSM, vascular smooth muscle; ND, not determined.

Table III

Kidney staining of agrin by MI-90, MI-91, GR-14, and Agr-131 on rat, human, and mouse renal tissue. GBM, glomerular basement membrane; TBM, tubular basement membrane; MM, mesangial matrix; BC, Bowman's capsule; PTC, peritubular capillaries; VSM, vascular smooth muscle; ND, not determined.
whereas in most other renal BMs predominantly a truncated agrin isoform is found that misses (part of) the C-terminal region.

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