Antibody Specificity of Human Glomerular Basement Membrane Type IV Collagen NC1 Subunits

SPECIES VARIATION IN SUBUNIT COMPOSITION

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NC1 subunits were purified from gel filtration pools of acid-extracted, collagenase-digested human glomerular basement membranes (hGBM). This methodology, which enriches 28-kDa monomers (M28) in the total digest, allowed purification of these monomers and 24-kDa (M24) and 26-kDa (M26) monomers free from dimers. Reactivity of these subunits with Goodpasture autoantibodies using immunoblotting of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and two-dimensional nonequilibrium pH gradient electrophoresis gels showed strong reactivity with the purified M28 subunits. Inhibition enzyme-linked immunosorbent assay, used to quantitate the reactivity of the autoantibodies using immunoblotting of sodium dodecyl sulfate-polyacrylamide gel electrophoresis and two-dimensional gel analysis of bovine and sheep basement membranes (hGBM, shGBM), and is exposed by urea denaturation which is enhanced by acid treatment. This is supported by their presence or absence in different species and tissues, as well as biochemical differences from the M24/26 monomers which presumably are derived from α1(IV) and α2(IV) collagen chains.

Type IV collagen has been shown to be a triple-helix molecule composed of two genetically distinct collagen chains, α1(IV) and α2(IV) (1). These collagen molecules are integrated into basement membranes in an unprocessed form, unlike interstitial collagens. Using the EHS tumor as a basement membrane model, it has been shown that a junctional region (7S collagen) is formed by covalent interaction of four NH₂-terminal propeptide trimers (2). The COOH-terminal propeptide trimers form a globular domain (NC1) which interacts via covalent bonding and noncovalent associations to form a hexameric structure (3) which is released by digestion with bacterial collagenase and has a molecular mass of 160-kDa. Rotary shadowing of interacting type IV collagen molecules formed the basis of the network model of basement membrane type IV collagen (4). More recent studies suggest a somewhat greater complexity of molecular interaction (5).

When EHS NC1 hexamers obtained from collagenase digests of tumor matrix were run by SDS-PAGE, a dimer/subunit composition was found. Two monomers of 26 and 24 kDa (M26 and M24, respectively) representing the α1(IV) and α2(IV) propeptides form dimers by reducible covalent linkage of the monomers (4). Examination of human glomerular basement membrane (hGBM) NC1 (6) and bovine GBM (bGBM) (7) using SDS-PAGE showed additional 28-kDa monomers of slightly higher mass (M28). Two-dimensional gel electrophoresis using NEPHGE in the first dimension and SDS-PAGE in the second dimension (8) separated the 28-kDa monomers into two components, one migrating to pH > 9.0 (M28++) and the second to pH 7.0 (M28±). The major dimers corresponded in pH to the M26 and M24 dimers, but additional dimers were present at pH > 9.0 and pH 7.0 (D54++ and D54±). Two-dimensional gels of EHS NC1 showed only the M26 and M24 monomers and their corresponding dimers (9).

The NC1 region of the type IV collagen molecule has been proposed as the site of the Goodpasture antigen based upon activity of immunoblotted NC1 dimers and monomers from...
collagenase-digested bGBM (7, 10), hGBM (6, 8, 9, 11), human placenta (12), and EHS tumor (9) with Goodpasture anti-GBM autoantibodies. When type IV collagen is isolated from bGBM and hGBM by guanidine extraction in the presence of reducing agents, followed by collagenase digestion, a major 30-kDa band by SDS-PAGE is observed which had identical mobility to that of the reduced NC1 monomers (13). The NC1 components isolated from collagenase digests of hGBM, in comparison with those derived from the EHS sarcoma (9), demonstrate greater reactivity against Goodpasture anti-GBM antibodies by inhibition ELISA assay. Immunoblotting of two-dimensional gels demonstrates that Goodpasture autoantibodies are directed primarily against the M28** monomer and the additional pH > 9.0 dimers found in the hGBM, although reactivity is demonstrated with all subunits. Immunoblots of bGBM NC1 show greatest reactivity with the high molecular weight monomer (M2*) (10). When hGBM is extracted with 0.5 M acetic acid prior to digestion with collagenase, the M28** monomer is enriched in gel filtration fractions along with the M28* monomer. RP-HPLC purification of these pools provides a reasonably pure preparation of M28 monomers. Increased reactivity of Goodpasture autoantibodies towards the two-dimensional gels of hGBM extracted with urea at low pH shown in this laboratory (14) demonstrates the role of denaturation as well as salt bond disruption in revealing the Goodpasture epitope.

The purpose of the present study was to analyze RP-HPLC-purified Goodpasture antigens from extracted hGBM by immunoblotting of SDS-PAGE and two dimensional gels and by inhibition ELISA assay. Additionally, we have shown that M28 monomers and D54 dimers appear to be unique to hGBM, we have examined GBM from bovine (hGBM) and sheep kidneys (shGBM) to determine if these components are present and establish whether these are unique to all kidney basement membranes. RP-HPLC-purified NC1 components obtained from these preparations were compared with hGBM and EHS NC1 using two-dimensional gel electrophoresis. Of particular interest was the comparison of the Goodpasture-reactive M2* component described by Butkowski et al. (10) in hGBM NC1 with the M28* and M28** monomers found in hGBM. We were also interested in defining the specificity of antibodies eluted from the kidneys of sheep immunized with whole hGBM (Steblay nephritis) (15) since this model has been compared with Goodpasture syndrome.

**EXPERIMENTAL PROCEDURES**

**Materials**—Nitrocellulose sheets were purchased from Schleicher & Schuell. Type VII collagenase was purchased from Sigma. Vydak TP201 C18 was obtained from Alltech. Peroxidase-labeled goat anti-sheep IgG (Fab')2 were purchased from Cappel/Cooper Laboratories. Normal human kidneys, which had been harvested but not used for transplantation, were obtained from patients with traumatic deaths. The tissues were provided by the National Diabetes Research Interchange after prompt freezing in buffer containing protease inhibitors.

The research was approved by the University of Minnesota Committee on the Use of Human Subjects in Research. Normal bovine and sheep kidneys were obtained from the University of Minnesota School of Veterinary Medicine.

**Baseement Membrane Isolation, Extraction, and Collagenase Digestion**—Human, sheep, and bovine glomeruli were isolated on ice in PBS with protease inhibitors as previously described (16). GBM was obtained by ultrasonic disruption in 1 M NaCl of whole glomeruli which had been separated by size-selective sieving of minced cortex. The sonicated GBM was passed through a 300-mesh screen to remove Bowman's capsules and tubular debris and washed three times using PBS with inhibitors. The GBM was treated twice at 4 °C with 0.5 M NaCl for 24 h with inhibitors as described elsewhere.2 After centrifugation at 11,000 × g, the pelleted GBM were then extracted in the presence of phenylmethylsulfonyl fluoride, N-ethylmaleimide, and pepstatin A using 0.5 M acetic acid for 72 h at 4 °C. Following centrifugation at 11,000 × g, the GBM extraction was applied to a 1.5 M Tris, 0.2 M NaCl, 0.1 M CaCl2, pH 7.4, for collagenase digestion. Ten units/mg protein of bacterial collagenase was used to digest the GBM for 24 h at 37 °C. Collagenase digestion of EHS tumor was obtained as previously described (9).

**Gel Filtration and Reverse-phase High Performance Liquid Chromatography**—Collagenase digests of extracted hGBM, shGBM, bGBM, and EHS tumor were separated on a Bio-Gel A-0.5 column (1.5 × 120 cm) equilibrated in 0.05 M Tris, 0.2 M NaCl, 0.1 M CaCl2, 0.02% sodium azide, pH 7.4, with constant monitoring at 280 nm using a UV Cord Model 213B spectrophotometer (LKB). Pools of column fractions were dialyzed against distilled water and lyophilized.

**Electrophoretic Analysis—** SDS-PAGE was performed in a Bio-Rad slab gel electrophoresis unit, using 8-15% linear gradient gels in a discontinuous buffer system (19). Unreduced samples from gel filtration pools were applied using 20-40 µg/lane while those from RP-HPLC were applied at 10-20 µg/lane. Low molecular weight standards dissolved in SDS sample buffer were used at 9 µg/lane.

Two dimensional gel electrophoresis was performed using NEPHGE in the first dimension (20) followed by 8-18% gradient SDS-PAGE in the second dimension as previously described (9).

NEPHGE tube gels were electrophoresed for 2750 V-h, frozen immediately in SDS sample buffer, and stored at −20 °C. Prior to second dimension separation, the gels were equilibrated for 30 min at room temperature in SDS sample buffer with gentle rocking. All gels were stained with Comassie Blue.

**Amino Acid Analysis**—Amino acid analysis was performed on a Dionex D-300 analyzer. Samples were dissolved in constant boiling HCl, sealed under a vacuum, and hydrolyzed at 105 °C for 24 h prior to analysis.

**Immunoblotting**—Antibody autoantibodies from serum 18 (14) were used for these studies. Sheep anti-GBM antibodies used in immunoblotting were obtained from the renal eluate of Steblay nephritis (SN) kidneys (15). The sheep immunization, kidney perfusion, and antibody elution using low pH citrate as well as additional purification steps for SN antibody eluate have been previously described (21) and kindly provided by K. Jeraj (University of Texas Health Science Center, Houston, TX).

**Immunochromy**—Immunoblotting followed the procedure previously described (22). Proteins were electrophoretically transferred to 0.45 µm nitrocellulose sheets from SDS-PAGE gels (25) using 200 mA constant current at 4 °C for 2 h or overnight. Goodpasture anti-GMB antibodies or eluted SN antibodies at a 1:20 dilution in 0.05 M Tris, 0.2 M NaCl, 1% bovine serum albumin, pH 7.4, were allowed to react with the blotted proteins for 1 h at room temperature. Peroxidase-labeled sheep anti-human IgG (Fab')2 were used as the secondary antibodies at a 1:500 dilution in the above buffer. After a 45-min incubation at room temperature and appropriate washing, the bound peroxidase-labeled secondary antibody was reacted with the substrate 3,3′-diaminobenzidine (acid form) using 20 µmol of the Tris buffer without sodium azide. The H2O2 was used with the substrate, and the reaction was terminated by washing in distilled water.

Inhibition ELISA assays were performed using RP-HPLC-purified unextracted hGBM NC1 as the coating antigen at 4 µg/ml in 6 µM guanidine HCl, 0.05 M Tris, 0.2 M NaCl, pH 7.4, using 200 µl/well. The plates were incubated overnight at room temperature. Goodpasture anti-GBM autoantibodies were inhibited with increasing concentrations of selected RP-HPLC-purified antigens as previously described (9). The antibodies were diluted 1:160 in 0.05 M Tris, 0.2 M

NaCl, pH 7.4, with 0.6 M guanidine HCl and reacted overnight at 4°C with inhibiting antigens in the same buffer. The coated plates were washed with PBS/Tween and the inhibited primary antibodies were incubated with antigen for 1 h at 37°C. The wells were washed three times with PBS/Tween and reacted with affinity purified peroxidase-labeled sheep anti-human IgG (Fab')₂ for 45 min at room temperature. After three washes, the substrate, ortho-phenylenediamine dihydrochloride, was added and the optical density was read at 450 nm using a Titertech Multiscan spectrophotometer (Flow Laboratories). Data were calculated as percent of the total absorbance obtained using RP-HPLC-purified 7S collagen (9) as a negative control with no inhibition, and zero absorbance representing complete inhibition.

RESULTS

Separation and Immunoreactivity of NC1 Components—
SDS-PAGE of Bio-Gel A-0.5 m gel filtration pools of hGBM NC1 pool III and hGBM M28 enriched pools IV and V are shown in Fig. 1A. Immunoblotting of these pools using Goodpasture anti-GBM autoantibodies is seen in Fig. 1B. M28-reactive material was found in pool III with NC1 dimers and monomers, the typical finding in hGBM. Pool IV and V contained an enrichment of GP-reactive M28 subunits, pool V being mostly free of dimeric material. Gel filtration of collagenase digests of extracted shGBM and bGBM gave results identical to those with hGBM. SDS-PAGE showed an increase in M26 and M24 monomers in pools IV and V when compared to the equivalent hGBM pools (not shown). Antigenic hGBM components were further purified using RP-HPLC; NC1 pool III from hGBM eluted at 33% acetonitrile using a 4%/min gradient (Fig. 2A). Similar NC1 profiles with varying amounts of unrelated peaks were found using pool III from shGBM and bGBM NC1. hGBM pool IV contained M28 monomers eluting at 35% CH₃CN (Fig. 2B) while M28 monomers free from dimers could be obtained from pool V (not shown). All active M24/26 monomers were separated from hGBM pool III at 31-33% CH₃CN using a 4%/min gradient (Fig. 2C). Various RP-HPLC pools were examined by SDS-PAGE (Fig. 2, insets) and immunoblotted using Goodpasture antibodies to track the separation of Goodpasture antigen NC1 (Fig. 3A).

The RP-HPLC-purified hGBM antigens were examined by immunoblotting two-dimensional gels using Goodpasture autoantibodies. The separation of hGBM pool III components using a 4%/min CH₃CN gradient is further demonstrated with separation of M24/26 monomers in the first (31-33% CH₃CN HPLC) pool (Fig. 3B) from D43/52 and M28 components in the second (33-35% CH₃CN HPLC) pool (Fig. 3C). Of note, minimal M26 material trailed into the second 33-35% CH₃CN RP-HPLC pool while M24/26 monomers were purified without dimers. The two-dimensional immunoblot of hGBM pool V (Fig. 3D) purified using a 4%/min CH₃CN gradient showed a predominance of M28⁺⁺⁺ and M28⁺⁺ monomers. hGBM NC1 prior to separation is shown for comparison (Fig. 3E).

Inhibition ELISA assay was performed using the RP-HPLC-purified reactive material. M24/26 monomers and D43/52 dimers with M28 monomers obtained from ½%/min CH₃CN separation of pool III and M28 monomers obtained from pool V separated using 4%/min CH₃CN were used to inhibit the reactivity of Goodpasture autoantibodies to hGBM pool III NC1. RP-HPLC-purified NC1 was also used as a positive control antigen to inhibit Goodpasture reactivity. Purified 7S collagen did not inhibit Goodpasture autoantibodies and therefore served as a negative control to establish values for 100% absorbance (no inhibition) at all concentrations (Fig. 4). It was shown that increased inhibition occurred as the concentration of M28 material increased in the samples, M24/26 components showing less ability to inhibit the antibodies than the plating antigen (NC1) which contained a small amount of M28. Pool III D43/54 and M28 components showed better inhibition than NC1, while purified M28 showed a 10-fold greater ability to give 50% inhibition than NC1 or the M24/26 monomers. From the slopes of all inhibition curves it appears that complete inhibition could ultimately be achieved if high enough concentration of each component was used.

Amino acid analysis of RP-HPLC-purified components obtained from collagenase digests of extracted hGBM are shown in Table 1 with comparison to corresponding EHS NC1 components. M24/26 monomers from hGBM and EHS are similar, with minimal variation in proline, glycine, and methionine content. D43/52 dimers from the two sources are also similar; the concentration of M28 in hGBM D43/52 dimers is not significant enough to affect the analysis of the dimers. M28 monomers show a distinctly different composition from the M24/26 monomers, with increased phenylalanine, lysine, leucine, and, most notably, hydroxylysine and decreased 4-hydroxyproline, valine, and histidine.

Fig. 2. A, RP-HPLC elution of hGBM NC1 dimers and monomers from gel filtration pool III. A 4%/min gradient of CH₃CN was used to elute samples of 150 µg. Similar profiles and SDS-PAGE were obtained using bGBM, shGBM, and EHS NC1. B, elution of monomers from gel filtration pool IV using a 4%/min gradient CH₃CN. C, RP-HPLC elution using ¼%/min CH₃CN gradient of hGBM gel filtration pool III (250 µg) containing the D43/54 dimers and M24/26 monomers. Insets are SDS-PAGE gels of pools as indicated, stained with Coomassie Blue. Abs, absorbance.

Fig. 1. A, SDS-PAGE gels of Bio-Gel A-0.5M column fractions stained with Coomassie Blue. Lane 1, standards: albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), and soybean trypsin inhibitor (20.1 kDa). Lane 2, pool III NC1; lane 3, pool IV monomers; lane 4, pool V monomers. B, Western blot of the SDS-PAGE gel reacted with Goodpasture autoantibodies. Lane 5, pool III NC1; note the enrichment of M28 in pools IV and V (lanes 6 and 7).
Antibody Specificity of Human Collagen NC1 Subunits

Fig. 3. A, Western blots of Goodpasture-reactive NC1 components. Reactive material further purified using RP-HPLC with 4%/min then 1%/min CH$_3$CN gradients as indicated and shown in Fig. 2. Note the very reactive M28 monomers enhanced in pools IV and V and the separation of active M24/26 monomers using 1%/min CH$_3$CN. B–E, Western blots of two-dimensional gels using NEPHGE in the first dimension followed by SDS-PAGE. RP-HPLC-purified antigens were reacted with Goodpasture autoantibodies. B, M24/26 pool III monomers obtained from 1%/min CH$_3$CN gradient separation (31–33% CH$_3$CN pool). C, D43/52 pool III dimers with M28 from 4%/min CH$_3$CN gradient separation (33–35% CH$_3$CN pool). D, Pool V M28 monomers separated using a 4%/min CH$_3$CN gradient. E, hGBM NC1 pool III containing all the active hGBM NC1 dimers and monomers separated using a 4%/min CH$_3$CN gradient. Note the reactivity of the M28 and D54 components of pH > 9.0 (M28+++ and D54+++) which are difficult to detect by Coomassie staining (not shown).

Fig. 4. Inhibition ELISA assay using RP-HPLC-purified antigens. The assay was plated with 4 µg/ml hGBM pool III, and Goodpasture autoantibodies were inhibited by increasing concentrations of RP-HPLC-purified antigens obtained from hGBM NC1 pool III. Data were plotted as percent of the total absorbance obtained. RP-HPLC-purified 7S collagen was used as a negative control to establish 100% absorbance with no inhibition at all concentrations (not shown). Note the rise in inhibition as the content of M28 components increases. Abs, absorbance.

using a 4%/min CH$_3$CN gradient was examined by two-dimensional gel electrophoresis. bGBM NC1 (Fig. 5A) was similar to hGBM NC1 (Fig. 5C) with the M28' monomer and the M24/26 monomers. There was less mass difference between the latter components, both being approximately 26 kDa. bGBM NC1 monomers were predominant over dimers compared to hGBM which had more dimer, and EHS NC1 which is mostly dimer (Fig. 5D). The bovine neutral D52 dimer had a higher apparent mass than its hGBM counterpart (D43 neutral), and the weakly cationic D54' dimer was not present. Of significance is the absence of the M28' monomer from bGBM NC1 which is the most reactive component in the hGBM with Goodpasture autoantibodies. This was confirmed by two-dimensional immunoblots (not shown). shGBM NC1 (Fig. 5B) looked most similar to hGBM by two-dimensional gel analysis. The proportion of dimer to monomer

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TABLE I
Amino acid analysis in residues/1000

compared to hGBM which had more dimer, and EHS NC1 which is mostly dimer (Fig. 5D). The bovine neutral D52 dimer had a higher apparent mass than its hGBM counterpart (D43 neutral), and the weakly cationic D54' dimer was not present. Of significance is the absence of the M28' monomer from bGBM NC1 which is the most reactive component in the hGBM with Goodpasture autoantibodies. This was confirmed by two-dimensional immunoblots (not shown). shGBM NC1 (Fig. 5B) looked most similar to hGBM by two-dimensional gel analysis. The proportion of dimer to monomer
and molecular weights of the dimers were similar to hGBM but shGBM M24/24 monomers more closely resembled hGBM monomers with less variation in molecular weight. A presumably unrelated Coomassie staining component (approximately 28 kDa) appeared at pH 9.0 which was not as reactive with Goodpasture autoantibodies as it was with Coomassie Blue but strongly with Goodpasture autoantibody (see Fig. 3E).

When hGBM NC1 was separated by two-dimensional electrophoresis and immunoblotted with SN antibodies eluted from a sheep kidney with Steblay nephritis, a staining pattern distinct from Goodpasture autoantibodies was observed (Fig. 6). Predominant reactivity was seen with the weakly cationic M28' monomer and D54' dimer, slight reactivity with D43/52 and D43/54' dimers when the hGBM was extracted with acid prior to collagenase digestion. The M28 monomers (M28' and M28*) can be enriched by prior extraction of the hGBM with acid before collagenase digestion and appear in several gel filtration peaks, as well as in the NC1 hexamer peak.

Although it seems that the major Goodpasture-reactive component in hGBM NC1 is the M28** monomer, we have shown that these autoantibodies also react less extensively with other NC1 dimers and monomers. Since the latter represent separate gene products, it is not apparent why Goodpasture antibodies also recognize the NC1 components; reactivity towards other NC1 components may occur via shared epitopes present in decreased quantities or cross-reactivity of similar epitopes.

Isolation of NC1 components from acid-extracted hGBM and shGBM confirmed the presence of reactive M28 monomers in the NC1 preparations; however, overall reactivity with GP autoantibodies was markedly reduced in these other species. Two-dimensional gels of RP-HPLC-purified bovine and sheep NC1 showed the M28* component as well as the D43/52 dimers and M24/26 monomers, but dimer/monomer ratios varied between these species. Molecular weight variation of some dimers exists between different species. Neither bovine nor sheep GBM NC1 contained M28** monomer. EHC NC1 does not contain M28** or M28* monomers nor their corresponding dimers (9), and SDS-PAGE of NC1 isolated from human placenta and bovine aorta (12) suggests a subunit composition more like the EHS. The M28* monomer appears to be common to GBM and, though less abundant, is also found in bovine lens capsule, while the very cationic most reactive M28*** appears, thus far, only in hGBM. Since Goodpasture syndrome as it affects the kidney is primarily a disease of the human glomerulus, the target antigen is likely the M28*** cationic monomer. Bound antibodies eluted from the kidneys of SN sheep induced by immunization with hGBM were reacted by two-dimensional gel immunoblotting with hGBM NC1. This showed primary reactivity with M28* monomer and D54* dimer, slight reactivity with D43/52 and D43/54' dimers with M28 monomers and D54 dimers when the hGBM was extracted with acid prior to collagenase digestion.

**DISCUSSION**

M28 monomers of hGBM NC1 are of considerable interest since these components either contain more Goodpasture autoantibody binding sites or have sites with higher affinity for these antibodies than the other NC1 components. Examination of two-dimensional immunoblots shows the greatest reactivity with the very cationic M28*** monomer and lesser reactivity to the weakly cationic M28** monomer and other NC1 components. Inhibition ELISA assay showed a trend of increasing inhibitory capacity using RP-HPLC fractions of hGBM NC1 containing M28 components. hGBM NC1 components that react with Goodpasture autoantibodies have been separated taking advantage of their different properties. NC1 dimers from gel filtration pools could be separated by RP-HPLC into fractions containing M24/26 monomers and M28 monomers.
M24/26 components, but no recognition of the M28*** component. These observations indicate that the SN nephritogenic antigen is the M28* monomer and D54* dimer; the latter, which appears in low quantities in hGBM, is most probably a dimeric form of M28*. Therefore, Steblay nephritis is a model pathogenetically similar but not identical to Goodpasture syndrome.

The differences in antibody recognition, charge, and presence or absence in different species and tissues suggest that the two M28 components have specific derivations. The weakly cationic M28* may represent the NC1 domain of an α chain distinct from α1(IV) and α2(IV) and possibly related to the new α(IV) chains recently described by Dixit et al. (24) in bovine anterior lens capsule. The very cationic M28*** protein is a unique hGBM component of unknown origin but with a well defined relationship to Goodpasture autoantibody and may be a specific unique derivatization of the M28* component. Variation in amino acid composition between M28 and M2* of Butkowski et al. (7, 10, 14, 25, 26), suggest that normally cryptic antigenic determinants in human kidneys and lung may be exposed.

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REFERENCES


4 EHS NC1 data of Weber et al. (3) show an average molecular weight of the two EHS monomers (24/26) obtained by sedimentation equilibrium ultracentrifugation and light scattering.
5 Values presented here for hGBM were obtained using SDS-PAGE gradient gels.
6 hGBM NC1 data of Butkowski et al. (10) was obtained using SDS-PAGE. The molecular masses for M1 and M2* as determined by sedimentation equilibrium ultracentrifugation were 28 and 32 kDa, respectively. Note that M2* is the equivalent of M28* in hGBM and does not reflect the contribution of M28*** in hGBM NC1.