Glycosylation and Size of IgA1 Are Essential for Interaction with Mesangial Transferrin Receptor in IgA Nephropathy

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Abstract. Transferrin receptor (TfR) has been identified as a candidate IgA1 receptor expressed on human mesangial cells (HMC). TfR binds IgA1 but not IgA2, co-localizes with mesangial IgA1 deposits, and is overexpressed in patients with IgA nephropathy (IgAN). Here, structural requirements of IgA1 for its interaction with mesangial TfR were analyzed. Polymeric but not monomeric IgA1 interacted with TfR on cultured HMC and mediates internalization. IgA1 binding was significantly inhibited (>50%) by soluble forms of both TfR1 and TfR2, confirming that TfR serves as mesangial IgA1 receptor. Hypogalactosylated serum IgA1 from patients with IgAN bound TfR more efficiently than IgA1 from healthy individuals. Serum IgA immune complexes from patients with IgAN containing aberrantly glycosylated IgA1 bound more avidly to TfR than those from normal individuals. This binding was significantly inhibited by soluble TfR, highlighting the role of TfR in mesangial IgA1 deposition. For addressing the potential role of glycosylation sites in IgA1-TfR interaction, a variety of recombinant dimeric IgA1 molecules were used in binding studies on TfR with Daudi cells that express only TfR as IgA receptor. Deletion of either *N*- or *O*-linked glycosylation sites abrogated IgA1 binding to TfR, suggesting that sugars are essential for IgA1 binding. However, sialidase and β-galactosidase treatment of IgA1 significantly enhanced IgA1/TfR interaction. These results indicate that aberrant glycosylation of IgA1 as well as immune complex formation constitute essential factors favoring mesangial TfR–IgA1 interaction as initial steps in IgAN pathogenesis.

IgA nephropathy (IgAN), the most common glomerulonephritis and a major cause of renal failure worldwide, is characterized by the presence of IgA1 deposits in the mesangium (1, 2). The mechanism involved in mesangial deposits and proliferation, characteristics of the initial phase of IgAN, is a major issue in understanding the development of this disease. Enhanced (two- to threefold) levels of serum IgA observed in patients with IgAN cannot fully explain IgA deposition, because other IgA-associated diseases, such as AIDS and IgA myeloma, also have increased levels of plasma IgA that do not always result in IgAN. An extrarenal origin of IgAN is suggested by recurrence of IgA1 deposits after renal transplantation, indicating that circulating factors are crucial (3, 4). It is now accepted that structural abnormalities of IgA1 are associated with the disease development (4). Patients with IgAN display two major alterations in the IgA system: (*1*) increased levels of circulating IgA-containing immune complexes (5–7) and (*2*) the generation of abnormally glycosylated IgA1 (8). IgA1 has heterogeneous *O*-glycans moieties in its hinge region (three to five *O*-linked carbohydrate chains). In IgAN, these *O*-glycans can be found incompletely galactosylated (7).

Several mechanisms may be responsible for the formation of IgA1-containing immune complexes in IgAN. One of the proposed mechanisms is based on the presence of hypogalactosylated IgA1 in serum that exposes terminal *N*-acetyl-D-galactosamine (GalNAc) generating neo-epitopes that are recognized by naturally occurring IgG and IgA1 antibodies, inducing circulating immune complexes (CIC) (8). The presence of abnormal *O*-glycans is also related to IgA1 stability in solution, as these molecules are prone to autoaggregation (9). Another mechanism implicated in IgA-complex formation is based on the enhanced binding of IgA1 from sera of patients with IgAN to FcαRI (10) that induces the release of a soluble form of FcαRI (M_r 50 to 70 kD). The pathogenic role of these soluble FcαRI-IgA1 complexes was demonstrated by transfer experiments using mice transgenic for the human FcαRI that spontaneously develop IgAN (11). However, other forms of soluble FcαRI (M_r 30 kD) exists in normal individuals and are not involved in IgAN (12). Finally, sera of patients with IgAN

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may contain a variety of CIC that involve extracellular matrix proteins such as fibronectin and collagen (4, 13, 14).

IgA1 and IgA1-containing immune complexes binding to human mesangial cells (HMC) seem mediated by an IgA receptor(s) (15). IgA1 can bind to rat and human HMC in a dose-dependent manner, and this leads to IgA internalization and degradation (15–21). Binding of heat-aggregated IgA to HMC induces cell activation, mobilization of intracellular calcium storage, protein phosphorylation (22), and release of IL-6 and TNF-α (16). However, none of the known IgA receptors (CD89, the asialoglycoprotein receptor, and the polymeric Ig receptor) is expressed by HMC (15, 18–21, 23, 24). Recently, we reported that the transferrin receptor (CD71/TfR) could serve as a mesangial IgA1 receptor. Indeed, TfR binds IgA1 but not IgA2 (25), and its expression is enhanced in the mesangium of patients with IgAN and co-localizes with IgA1 deposits (25, 26).

In this study, we analyzed the role of glycosylation and the molecular form of IgA in the interactions with TfR expressed on HMC. Our results revealed that polymeric IgA1 but not monomeric IgA1 binds to TfR and that either TfR1 or TfR2 can participate in this binding. We showed that IgA1 isolated from serum of patients with IgAN and macromolecular IgA containing hypogalactosylated IgA1 present in serum from patients with IgAN showed enhanced binding to TfR expressed on HMC. Finally, we analyzed the role of IgA1 glycosylation in TfR binding by studying either the interaction of a variety of recombinant IgA1 protein lacking *N*-linked or *O*-linked glycosylation sites or the ability of deglycosylated IgA1 to bind TfR. All together, our data indicate that despite that *O*- and *N*-linked glycosylation sites of IgA1 are essentials, hypogalactosylation or desialylation of IgA1 promotes enhanced IgA1 binding to TfR.

Materials and Methods

Subjects

Sera from patients with IgAN (diagnosed by the presence of predominant IgA1 deposits in the mesangium associated with focal or diffuse mesangial cell proliferation; $n = 15$) and healthy, randomly selected subjects ($n = 15$) were studied. In each instance, informed consent was obtained from the donors for the use of blood samples for experimental purposes.

Antibodies and Reagents

The following mAb were used: A24 anti-TfR (γ2bκ) (25), MA712 anti-TfR (γ2a; Pharmingen, San Diego, CA), CH-EB6-8 (γ1κ) anti-IgA (American Type Culture Collection, Rockville, MD) (27), 1-155-1 (γ3λ) anti-IgA1, and 14-3-26 (γ2bκ) anti-IgA2 (28). Polyclonal anti-human transferrin (Sigma Chemical Co., St. Louis, MO) was coupled to activated Sepharose 4B beads (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's recommendations. Human myeloma IgA1 (Dou) was purified as described (25). IgA2λ (IgA2m[2]; Fel) was provided by Dr. J. Mestecky (University of Alabama at Birmingham, Birmingham, AL) and IgA2κ (IgA2m[2] Bel) was a gift from Dr. P. Aucouturier (Inserm E0209, St. Antoine Hospital, Paris, France). Fractions containing monomeric (m) and polymeric (p) IgA were prepared by size-exclusion chromatography on Superdex 200 columns through HPLC (Amersham; >99% pure). Transferrin-free IgA (purified by gel filtration and immunoabsorption through anti-transferrin Sepharose 4B columns) was biotinylated as described (25). Human serum IgG was purified by ammonium sulfate precipitation and DEAE ion exchange chromatography. Proteins were quantified by the BCA method according to the manufacturer's instructions (Pierce, Rockford, IL).

The generation and purification of recombinant human IgA1 (α1), IgA1 with the hinge region of IgA2 (α1 with α2 hinge), IgA2 with the hinge region of IgA1 (α2 with α1 hinge), IgA1 lacking the *N*-linked glycosylation sites (α1 with no N-CHO), and IgG1 have been previously described (29–31).

Modification of IgA glycans composition were obtained by enzymatic treatments of myeloma pIgA1 (Dou) using N-glycosidase F, O-glycosidase, neuraminidase from *Vibrio cholerae* (Roche Molecular Biochemicals, Mannheim, Germany), and β-galactosidase from bovine testes (Sigma-Aldrich) in accordance with the manufacturer's instructions and as described (8).

Cells

Primary HMC from single donors were purchased from a commercial source (Clonetics, San Diego, CA). Cells were cultured in RPMI 1640, supplemented with glutamine (2 mM), 5 μg/ml insulin, 20% FCS (Life Technologies, Gaithersburg, MD), 7 mM glucose, 50 U/ml penicillin, and 50 μg/ml streptomycin in a 7% CO_2 atmosphere. Cells were detached with 0.25% trypsin and 0.5% EDTA (Invitrogen, Carlsbad, CA). Trypsinization was no longer than 2 min at 37°C, and the reaction was stopped by addition of cold RPMI containing 10% FCS. Under these conditions, the cells were viable (>95%) and TfR expression was not affected as evaluated by flow cytometry using an anti-TfR mAb. Studies were performed using HMC from passages 4 to 8. Established Daudi cell line was obtained from American Type Culture Collection.

Expression and Purification of TfR1 and TfR2 Ectodomains

Soluble (s) versions of human TfR1 and TfR2 were expressed in a lytic baculovirus/insect cell expression system as described previously (32). Stability of soluble TfR was assessed by measuring its binding to holotransferrin. The construct for the irrelevant control soluble form of HLA-DR4 was provided by J.M. Fourneau (Necker Hospital, Paris, France).

Affinity Purification of IgA1 Fractions

IgA1 fractions were purified by jacalin affinity and anti-IgA1 chromatography. Briefly, jacalin was purified from jackfruit extracts by DEAE column as described previously (33) and coupled to Sepharose 4B beads (Pharmacia). Serum fractions were diluted in PBS and absorbed on jacalin-Sepharose columns, and lectin-binding proteins were eluted overnight with 0.5 M galactose as described (33). Serum IgA1 was also purified by affinity chromatography using anti-IgA1 Sepharose columns as described previously (8, 15, 34).

Immunofluorescence Analysis

Cells (0.25×10^6) were preincubated with 10 μl of human IgG (10 mg/ml) for 15 min on ice to mask FcγR. IgA binding was examined using an indirect immunofluorescence assay in which cells that were preincubated with human IgG were incubated with 10 μl of IgA (0.5 to 10 mg/ml) for 1 h on ice before washing and incubation with a biotinylated anti-IgA mAb (clone CH-EB6-8 that recognizes both IgA1 and IgA2) for 20 min at 4°C. After washes, Allophycocyanin-labeled streptavidin (Southern Biotechnology Associates, Birming-

ham, AL) was used as a developing reagent. In some experiments, biotinylated HPLC-purified IgA1 (free of transferrin) plus phycoerythrin-labeled streptavidin were used. For inhibition studies, biotinylated myeloma IgA or unlabeled IgA-containing circulating immune complexes were preincubated with soluble receptors at indicated concentrations for 1 h before addition to the cells. Immunofluorescence was finally analyzed by flow cytometry (FACScalibur; Becton Dickinson, NJ).

Confocal Microscopy

HMC were incubated for 30 min at 4°C to block membrane recycling and then labeled with biotinylated pIgA1 (0.5 mg/ml) for 60 min at 4°C. After washes, cells were incubated for 30 min with streptavidin phycoerythrin (Southern Biotechnology Associates; 1:50 dilution). Cells were washed and shifted to 37°C for different time points. HMC were fixed in 4% paraformaldehyde, quenched with 0.1 M glycine, blocked in PBS containing 2% FCS, and permeabilized with 0.005% saponin in PBS containing 0.1% BSA. TfR next were stained with the mAb A24 (10 μg/ml) for 30 min at 4°C. After washes, A24 mAb was revealed by a goat anti-mouse IgG antibody conjugated to Cy-5 (Jackson ImmunoResearch, West Grove, PA). Slides were examined with a confocal laser microscope system (LSM 510 Carl Zeiss, Oberkochen, Germany).

Fractionation of Serum

A total of 0.5 ml of serum was diluted in phosphate buffer (0.05 M Na_2SO_4, 0.02 M NaH_2PO_4 [pH 7.5]) (8), filtered, and separated by gel filtration through an S-300 Sephacryl column (Amersham Pharmacia) connected to an HPLC AKTA-basic automated liquid chromatography system (Amersham Pharmacia). The molecular size of each fraction was determined by the content of different serum proteins revealed by immunoelectrophoresis. Fractions of 1.0 ml were collected and analyzed. IgA concentration in the fractions was determined using a sandwich ELISA method.

ELISA

For determining IgA concentration in different column fractions, plates were coated with mAb CH-EB6-8, anti-IgA1, or anti-IgA2 mAb at 5 μg/ml in borate-buffered saline (BBS) for 2 h at room temperature. The wells were then washed twice in BBS containing 0.05% Tween and blocked for 2 h at room temperature in PBS containing 1% BSA and 0.1% sodium azide. Column fractions (diluted at 1:100) were incubated overnight at 4°C. A polyclonal anti-IgA alkaline phosphatase (AP) conjugate (Southern Biotechnology Associates) was used as developing antibody. The optical density at 450 nm was measured after addition of streptavidin-AP substrate (Sigma) following the manufacturer's instructions. Soluble TfR was measured in serum by ELISA using two mAb, clone MA712 from Pharmingen and clone A24 (25).

Gel Electrophoresis and Immunoblotting

SDS-PAGE and Western blotting were performed as described elsewhere (35, 36). Briefly, 20 μg of protein extracts was solubilized in SDS-sample buffer under nonreducing conditions at 100°C, subjected to electrophoresis in a 4% polyacrylamide gel, and electroblotted onto polyvinylidene fluoride membranes (Millipore, Bedford, MA). Membranes were blocked overnight with TTBS buffer (50 mM Tris-HCl, 150 mM NaCl [pH 7.4], 0.05% Tween 20) containing 4% BSA (wt/vol) at 4°C. For detecting IgA, strips were incubated with 1 μg/ml biotinylated anti-IgA mAb CH-EB6-8 in 1% BSA in TTBS. Alternatively, the presence of altered glycosylated IgA was verified using biotinylated Helix aspersa (HAA; EY Laboratories) at 1 μg/ml using the same conditions described above. Membranes were washed and then incubated with streptavidin-horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) at a 1:30,000 dilution in TTBS buffer containing 1% BSA. Blots were developed with West-Pico substrate (Pierce) according to the manufacturer's instructions. Band intensity was evaluated by NIH Image software and considered as increased when greater than the mean + 2 SD of control bands intensity.

Statistical Analyses

The results were analyzed by independent sample two-tailed *t* test. Results are presented as means ± SD.

Results

Polymeric but not Monomeric IgA1 Binds to TfR on HMC

We have previously shown, using a direct immunofluorescence assay based on biotinylated IgA preparations, that mIgA1 binds more than pIgA1 to TfR (25). Because our mIgA1 preparations contained minimal (<1%) amounts of transferrin, we reexamined the type of IgA form involved in TfR binding using an indirect immunofluorescence assay developed by a biotinylated anti-human IgA mAb (clone CH-EB6-8 that recognizes both IgA1 and IgA2 subclasses) and streptavidin-Allophycocyanin. We evaluated the binding of several types and forms of IgA, including mIgA1, pIgA1, mIgA2, pIgA2, and secretory IgA (SIgA) to HMC. HMC express TfR (25) but not the other known IgA receptors, including the CD89, the polymeric-Ig receptor (pIgR), and the asialoglycoprotein receptor (ASGP-R) (15, 18–21, 23). Under our experimental conditions, only pIgA1 binding differed significantly from control human IgG (Figure 1A). By contrast, using this method, we detected binding of both IgA1 and IgA2 to CD89 expressing cells (data not shown). The sensitivity of this method was evaluated by a dose-response curve of IgA binding. Figure 1B shows FACS histograms of a dose-response curve of pIgA1 binding. pIgA1 binding differs from the control IgG for values >0.5 mg/ml. Binding of pIgA1 to HMC is linear, dose-dependent, and not saturable up to 10 mg/ml (Figure 1C). The specificity of IgA1 binding to mesangial TfR was next verified by blocking mesangial binding of pIgA1 by the addition of soluble forms of TfR1 and TfR2 (sTfR1 and sTfR2) produced in a baculovirus/insect cells expression system. Preincubation of pIgA1 with sTfR2 at a soluble receptor: pIgA molar ratio of 1.5 inhibited by 55% the binding to HMC. Under the same conditions, sTfR1 inhibited IgA1 binding by approximately 50% (Figure 2A). The control soluble form of the unrelated HLA-DR4 molecule did not inhibit IgA binding to these cells. In addition, inhibition of pIgA1 binding to HMC using both sTfR1 and sTfR2 was dose dependent (Figure 2B). To address the specificity of IgA1-TfR interaction, we performed similar experiments using the Daudi cell line, cells that express TfR as the only IgA1 receptor (25). Both sTfR1 and sTfR2 at a soluble receptor:pIgA molar ratio of 1.5 inhibited pIgA1 binding (Figure 2C). Using anti-IgA immunofluorescence indirect assay, holotransferrin failed to inhibit pIgA1 binding to Daudi and HMC up to 1 mg/ml (data not shown).

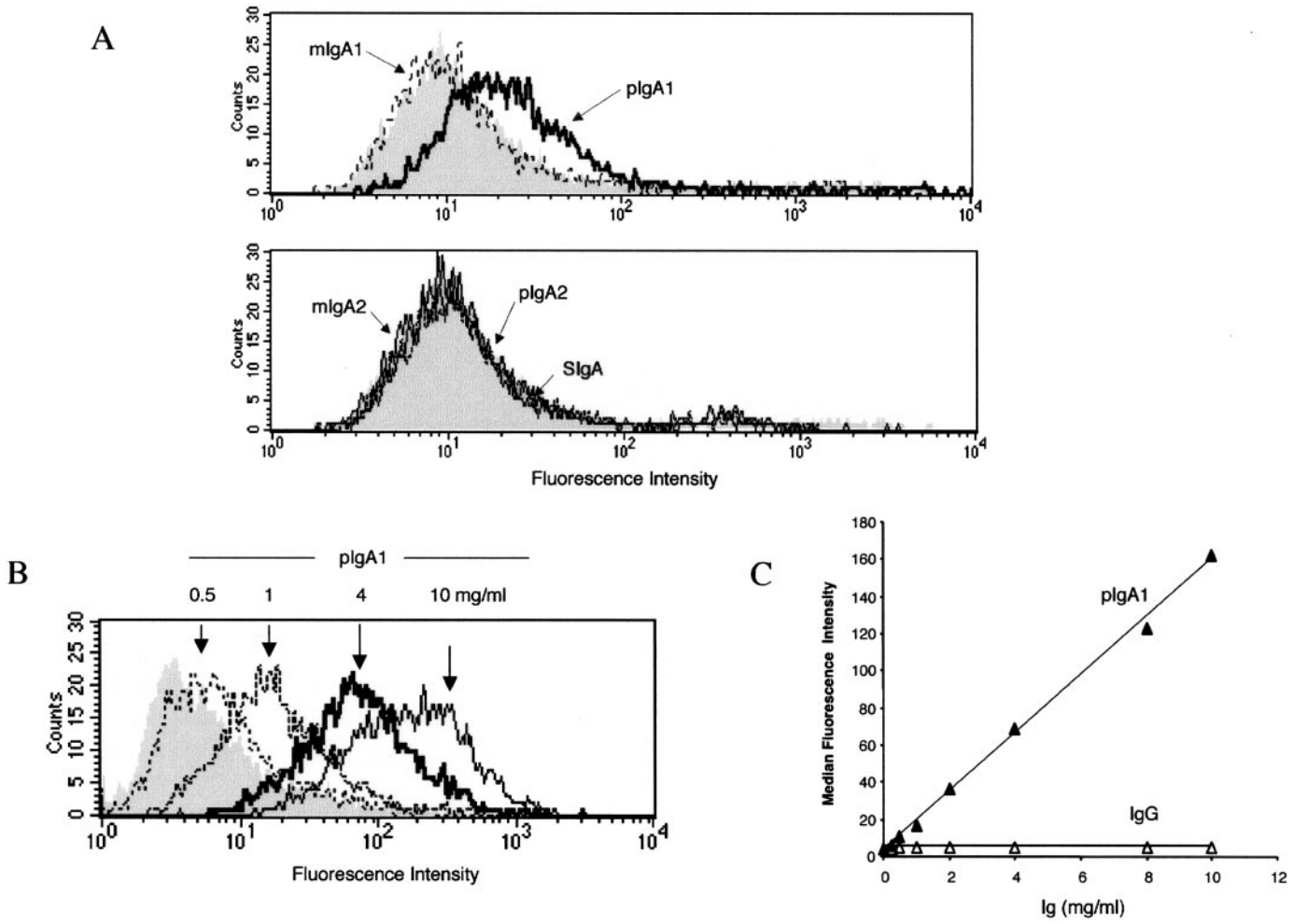

Figure 1. Polymeric IgA1 (pIgA1) but not monomeric IgA1 (mIgA1) binds to human mesangial cells (HMC). (A) Comparative immunofluorescence analysis of several types and forms of IgA including mIgA1, pIgA1, mIgA2, and pIgA2, and secretory IgA (SIgA) at 1 mg/ml. Human IgG (filled histogram) was used as negative control. IgA binding to HMC was evaluated using biotinylated anti-human IgA mAb (CH-EB6-8) and streptavidin-Allophycocyanin (APC) as the developing reagent. (B et C) pIgA1 binding is concentration dependent. (B) HMC were incubated with serial dilutions of pIgA1 and binding was detected by biotinylated CH-EB6–8 mAb followed by streptavidin-APC. (C) Fluorescence median intensities of FACS analyses of pIgA1 binding were plotted, and IgG was used as negative control.

These results indicate that pIgA1 binding to HMC is mainly mediated by the transferrin receptor.

pIgA1 Induces TfR Endocytosis and Co-localizes with Intracellular TfR Compartments

The transferrin receptor plays a key role in Fe uptake and is constantly recycled in the cell (37). This homodimeric molecule binds two Fe-loaded (holo) transferrin molecules at the cell surface and is internalized. Recycling vesicles containing the TfR:holotransferrin are acidified, and Fe dissociates from Tf. The remaining Tf-TfR complex remains associated at pH $\leq$6.4 and dissociates after recycling to the cell surface at pH 7.4 (37). Under physiologic conditions, no internalization of TfR is observed. However, this can be achieved by anti-TfR antibodies. Functional IgA receptors such as CD89 and the ASGP-R are internalized and degraded after binding of IgA-containing immune complexes (23). Therefore, we examined whether pIgA1 binding to TfR could mediate TfR endocytosis.

Cells stained with pIgA1 were allowed to endocytose, fixed, and stained intracellularly with the anti-TfR mAb A24. As shown in Figure 3, TfR was initially localized near the cell surface in recycling vesicles, which is a characteristic feature of TfR (37). After 5 min, pIgA1 co-localized with TfR at the cell membrane and in the recycling vesicles. After 15 min, TfR was found in intracellular compartments, where it co-localized with internalized pIgA1, indicating that pIgA1 induced endocytosis of TfR.

Binding of IgA Immune Complexes from Sera of Patients with IgAN to Cultured Mesangial Cells Is Mediated by the TfR

To determine the binding characteristics of immune complexes from patients with IgAN to cultured mesangial cells, we compared binding characteristics of macromolecular IgA purified from normal subjects and patients with IgAN with

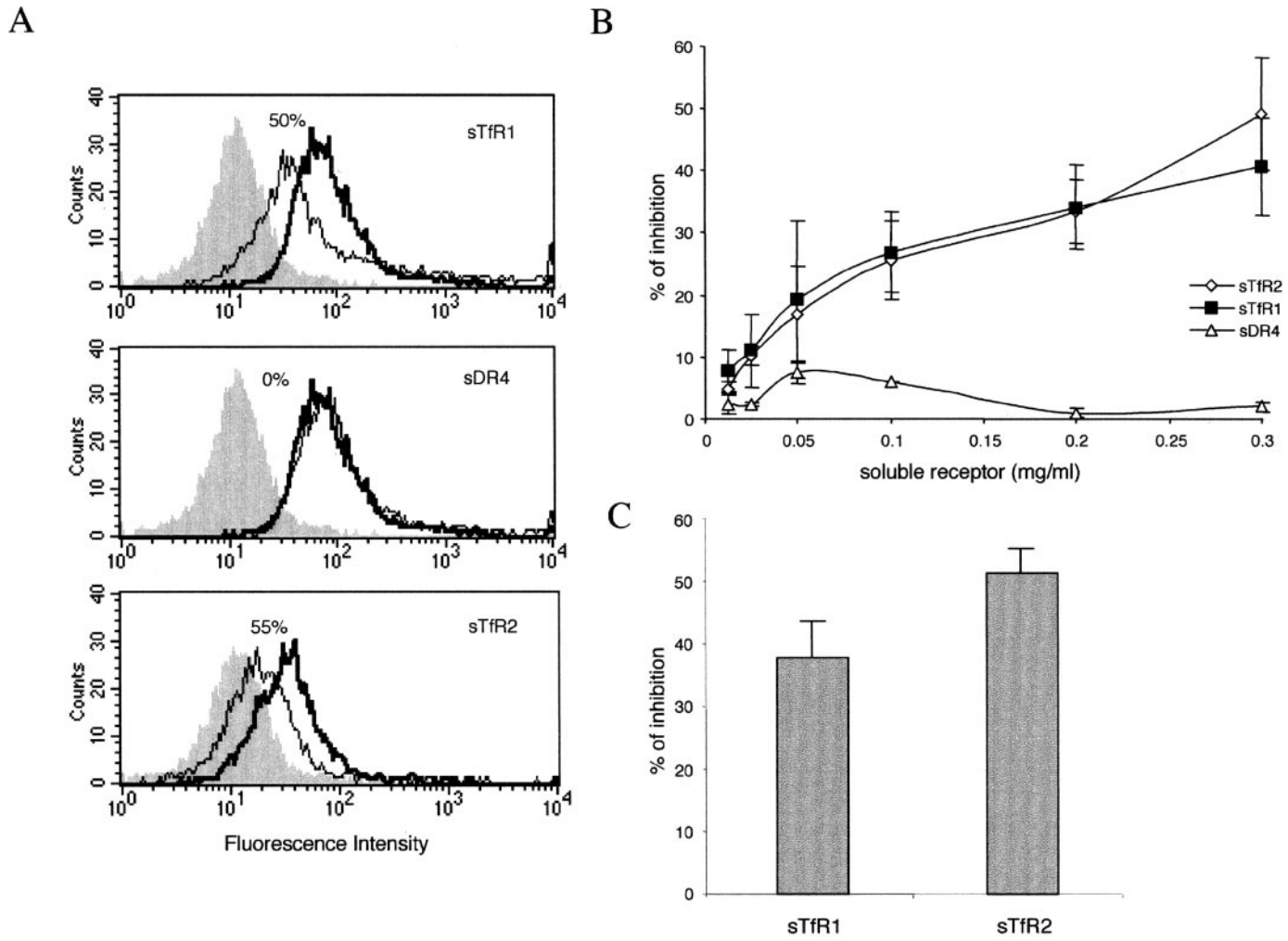

Figure 2. pIgA1 binding to HMC is inhibited by transferrin receptor (TfR) ectodomains. (A) Immunofluorescence analysis of the ability of the TfR1, TfR2, and HLA-DR4 ectodomains to block biotinylated pIgA1 binding on HMC. Biotinylated pIgA1 was purified by gel filtration through HPLC using Sephacryl S-200 columns and further passed through anti-transferrin beads to eliminate any residual transferrin. pIgA1 fractions (99.9% pure) devoided of were then used for binding experiments. In each panel, the heavy dark line indicates the control pIgA1 binding and the thin line indicates binding in the presence of the indicated soluble protein. Human IgG was used as a negative control of binding (filled histogram). (B) Soluble (s) forms of TfR1 and TfR2 inhibit pIgA1 binding to HMC in a dose-dependent manner. The results are the mean inhibition (± SD) of median fluorescence intensity over background of FACS analysis of three independent experiments performed as in A. (C) Soluble (s) forms of TfR1 and TfR2 inhibit pIgA1 binding to Daudi cells. Experiments were performed as in (B) with Daudi cells.

mesangial TfR. Sera from healthy control subjects and from patients with IgAN were fractionated using HPLC with a gel filtration column. Figure 4A shows a representative profile of resolution through gel filtration of normal and IgAN serum. Differences between the protein profiles of sera from control subjects and patients with IgAN occurred for high-molecular-weight (>850 kD) proteins. IgA distribution between these fractions was determined using an ELISA capture assay (Figure 4B). The amount of macromolecular IgA species was increased in sera from patients with IgAN. The polymeric nature of IgA was further confirmed by anti-IgA Western blot. Different fractions were separated in a nonreducing 4% SDS-PAGE and immunoblotted, and IgA was detected using a biotinylated monoclonal anti-IgA antibody (CH-EB6-8). IgA1 was predominant within these fractions, but a similar IgA1/IgA2 ratio was observed between control and patient fractions (not shown). As shown in Figure 4C, IgA from patients contained higher molecular weight pIgA than those from normal subjects. For addressing the question of whether IgA from patients contained abnormal *O*-glycans, the same protein amount of IgA-containing high-molecular-weight fractions from a patient and from a control subject were blotted with biotinylated HAA (a lectin that binds specifically to terminal GalNAc). Enhanced HAA binding was observed only in the polymeric IgA fractions from the patients with IgAN (Figure 4D).

We next addressed the characteristics of macromolecular IgA binding to mesangial TfR. The concentrations of macromolecular IgA from normal individuals and patients with IgAN were normalized, and these IgA preparations were incubated

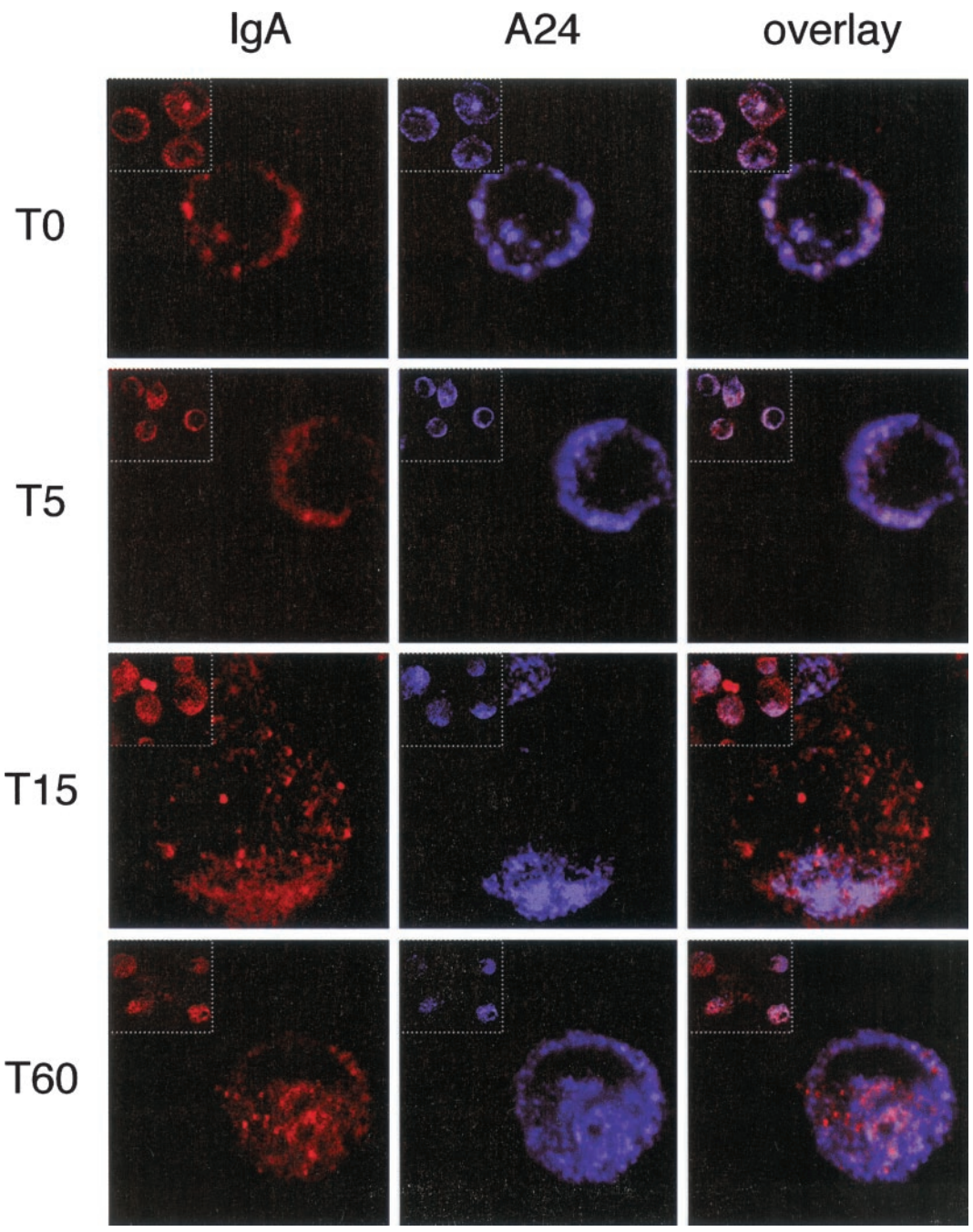

Figure 3. pIgA1 induces TfR endocytosis. Cell suspensions of HMC were incubated with 0.5 mg/ml biotinylated pIgA1 and further decorated with streptavidin-phycoerythrin (red) at 4°C. After washing, cells were shifted to 37°C for the time indicated. Cells were then washed, fixed, and permeabilized. TfR was then labeled with the mAb A24 and an anti-mouse-Cy-5 (blue).

with HMC followed by FACS analysis of IgA binding. Under these conditions, three of five samples of macromolecular IgA from sera of patients with IgAN showed increased binding to HMC (Figure 5A). The binding of macromolecular IgA to HMC was shown to be dose dependent (Figure 5B). These results confirm the data previously obtained by others ((15) showing that high-molecular-weight IgA from sera of patients with IgAN have enhanced binding to HMC. The specificity of IgA complexes binding to mesangial TfR was verified by inhibition of IgA binding to HMC by soluble forms of TfR1 and TfR2; sTfR1 and sTfR2 significantly inhibited IgA complex binding to HMC, whereas soluble HLA-DR4 did not (Figure 5C). Fractions containing low M_r IgA species (160 and 60-kD heavy chain) did not bind TfR (not shown).

Aberrantly Glycosylated IgA1 Binds More to TfR than Normal IgA1

Because IgA1 from sera of patients with IgAN had been shown to have altered *O*-linked glycosylation (7), we examined whether patient IgA1 has an enhanced binding capacity to TfR. IgA1 proteins were isolated from sera of healthy donors and patients with IgAN using a jacalin-Sepharose affinity column (abnormally glycosylated IgA can bind to some preparations of jacalin as reported (38)). The aberrantly glycosylated IgA was

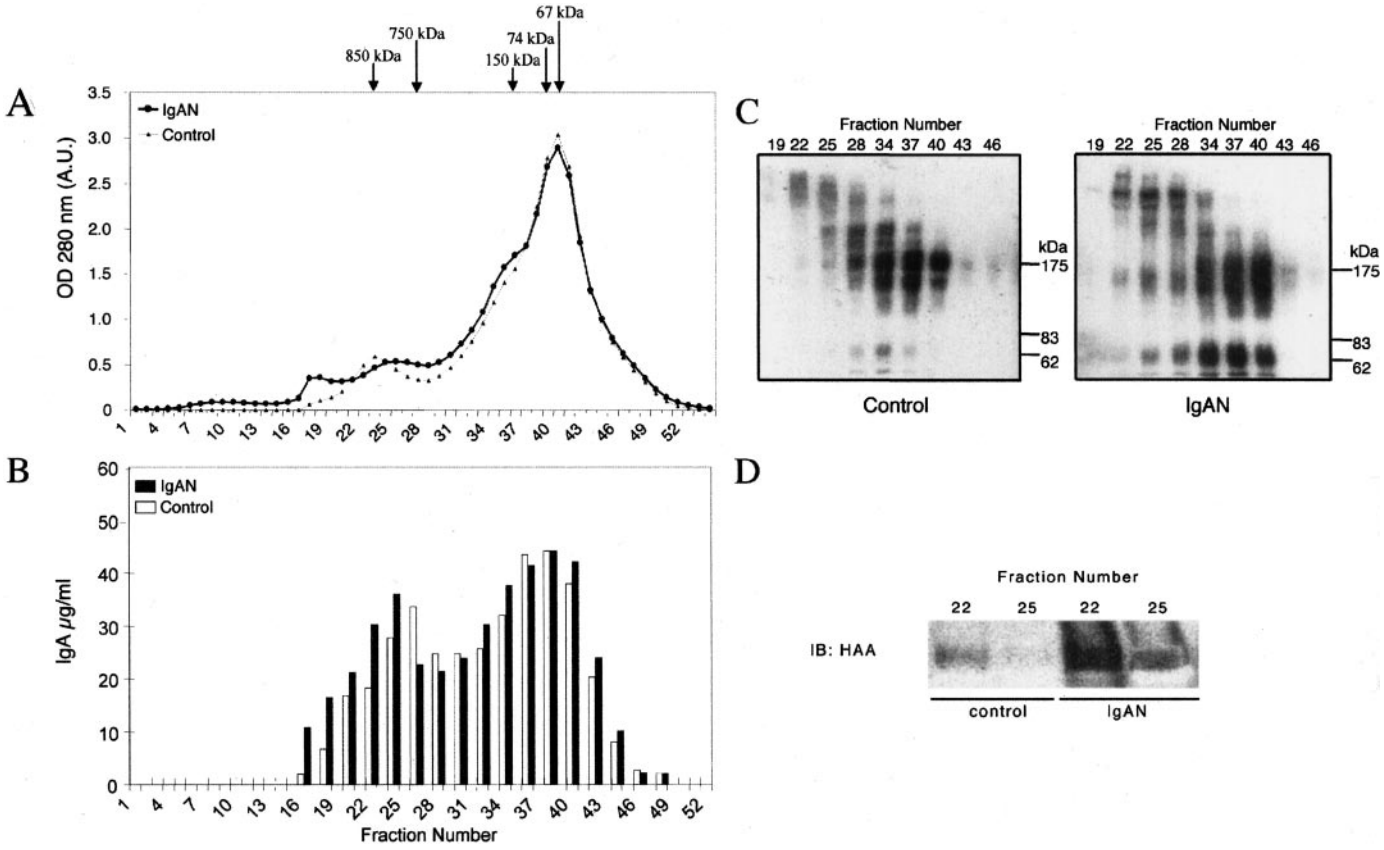

Figure 4. Purification of IgA complexes from patients with IgA nephropathy (IgAN) and healthy subjects. (A) Serum from patients with IgAN and healthy subjects were fractionated by gel filtration through HPLC using a Sephacryl S-300 column. Separated fractions were analyzed for both their protein (A) and IgA (B) content. Molecular masses of the calibrated column are indicated at the top of the figure. (C) Detection of different IgA sizes contained in each fraction by immunoblotting using biotinylated anti-IgA and streptavidin–horseradish peroxidase (HRP) as a developing reagent. Molecular weight markers are indicated in the right side. (D) Detection of abnormally glycosylated IgA in high-molecular-size fractions by immunoblotting using biotinylated-HAA and streptavidin-HRP as developing reagent.

detected by HAA binding. IgA fractions were normalized in their protein content and blotted using biotinylated HAA. Samples (seven of 15) from patients with IgAN had an enhanced HAA binding, indicating the presence of galactose-deficient IgA1 (data not shown). We therefore evaluated the ability of jacalin-purified IgA1 from patients with IgAN and control subjects to bind to HMC (0.5 mg/ml). A significant increase in IgA1 binding was observed with material from patients with IgAN (Figure 6A).

Daudi cells express TfR as the only IgA receptor (the only IgA receptor recovered from these cells by IgA affinity columns is the TfR) (25). Therefore, we next studied binding characteristics of patients' IgA1 to these cells as compared with control IgA. Figure 6B shows that jacalin-purified IgA1 from patients with IgAN at the same concentration (0.5 mg/ml) bound significantly more to Daudi cells than those from control subjects, confirming data obtained with HMC. Immuno-affinity purified IgA1 from both control subjects and patients with IgAN were also used in binding experiments on Daudi cells. Figure 6C shows that patient IgA1 have an enhanced binding to Daudi cells.

IgA1 Binding to TfR Is Dependent on the Presence of Both O- and N-Linked Glycans

Because aberrantly glycosylated *O*-glycans in the hinge region of IgA1 have been shown to be important in binding to mesangial cells in IgAN (15), we hypothesized that the *O*-linked carbohydrates in the hinge region of IgA1 could mediate the binding of IgA1 to TfR on HMC. To test this hypothesis, we studied the binding to TfR on Daudi cells of well-characterized recombinant IgA1, IgA2, IgA1 with IgA2 hinge region, IgA2 with IgA1 hinge region, and IgA1 lacking the *N*-linked glycosylation sites in the α heavy chains (31) (Figure 7A). The human IgA1 contains three to five *O*-linked glycans in a 26–amino acid hinge region located between domains Cα1 and Cα2, whereas IgA2, which has a 13–amino acid hinge region, lacks the *O*-linked glycans (7, 30) (Figure 7A). All proteins contain predominantly polymers with J chain (data not shown). Significant binding to TfR was observed only with wild-type IgA1. However, this binding was lower than that observed for polymeric myeloma IgA1 (Dou; Figure 7, B and C). IgA2 did not bind to TfR on Daudi cells, suggesting that the IgA1 hinge region is required for binding to TfR. In addition, IgA2 con-

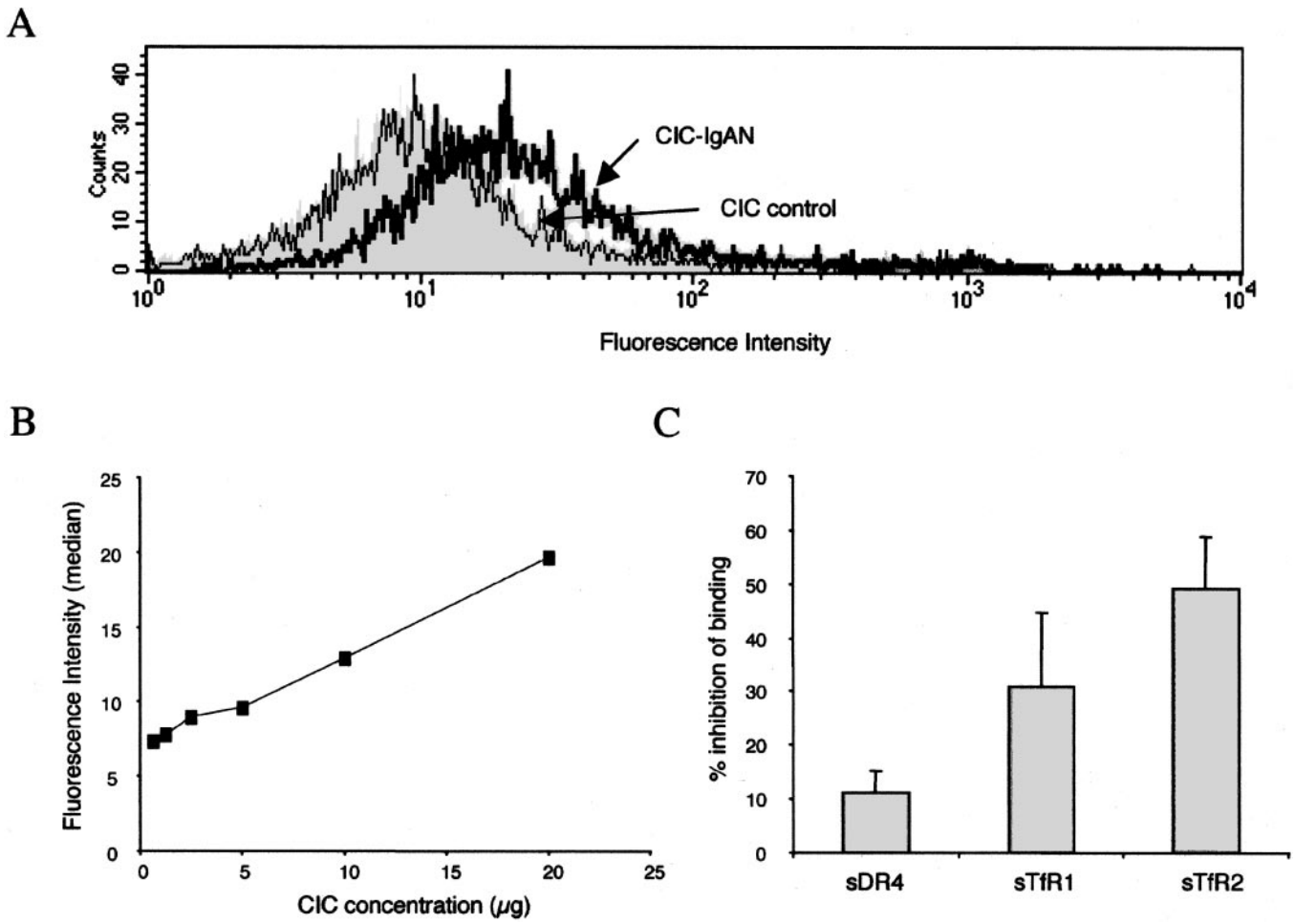

Figure 5. Binding of IgA-complexes from patients with IgAN to HMC is mediated by TfR. (A) HMC were incubated with 20 μg of IgA complexes (column fractions 22 to 25) from patients with IgAN and control subjects, and IgA binding to TfR was evaluated using biotinylated anti-human IgA mAB (CH-EB6-8) and streptavidin-APC as the developing reagent. (B) IgA complexes binding to HMC is concentration dependent. HMC were incubated with the indicated concentrations of IgA complexes from patients with IgAN, and IgA binding was evaluated by FACS analysis using biotinylated CH-EB6-8 mAb followed by streptavidin-APC. Median fluorescence intensities were plotted after subtracting values of each corresponding concentration of biotinylated IgG. (C) Soluble forms of TfR1 and TfR2 but not HLA-DR4 inhibit IgA complexes binding to HMC. IgA complexes were preincubated with soluble receptor at a 1.5 molar ratio. Complexes were then transferred to HMC, and IgA binding was evaluated as described above. Histograms represent mean ± SEM of three independent experiments.

taining the IgA1 hinge region did not bind to TfR, suggesting that IgA1 hinge region is not sufficient to promote binding. Remarkably, IgA1 either lacking *N*-linked glycosylation in the α heavy chains or lacking *O*-linked glycosylation did not bind to Daudi cells, indicating that both *N*- and *O*-linked glycosylation is required for IgA1 binding to TfR (Figure 7, B and C). Taken together, these results suggest that both *O*- and *N*-linked glycosylation in the context of IgA1 is required for building the IgA1 binding site to TfR.

Degalactosylation and Desialylation of IgA1 Enhances Its Binding Capability to TfR on Daudi and HMC

Altered glycosylated IgA present in the circulation of patients with IgAN have an enhanced affinity to mesangial cells (15, 17–19, 21). However, whether those IgA glycoforms are capable of interacting with TfR is unknown. Carbohydrate moieties of IgA were modified using different enzymatic treatments, and IgA1/TfR interaction was analyzed on Daudi cells that express the TfR as the only IgA receptor (25). Figure 8A shows that *O*-glycosidase abrogates IgA1 binding to TfR, whereas N-glycosidase has no major effect. By contrast, neuraminidase and neuraminidase plus β-galactosidase significantly enhanced IgA1 binding to TfR. Desialylated and degalactosylated IgA1 also have an enhanced binding to mesangial cells (Figure 8B). This enhanced binding was strongly inhibited by TfR ectodomains, indicating that TfR is mediating the interaction between aberrant *O*-linked glycosylated IgA1 and HMC (Figure 8B).

Levels of Soluble TfR Are not Enhanced in Serum of IgAN Patients

Human serum usually contains a truncated form of the TfR that circulates in the blood complexed to transferrin (39). To investigate whether IgA1 binding to TfR could induce secre-

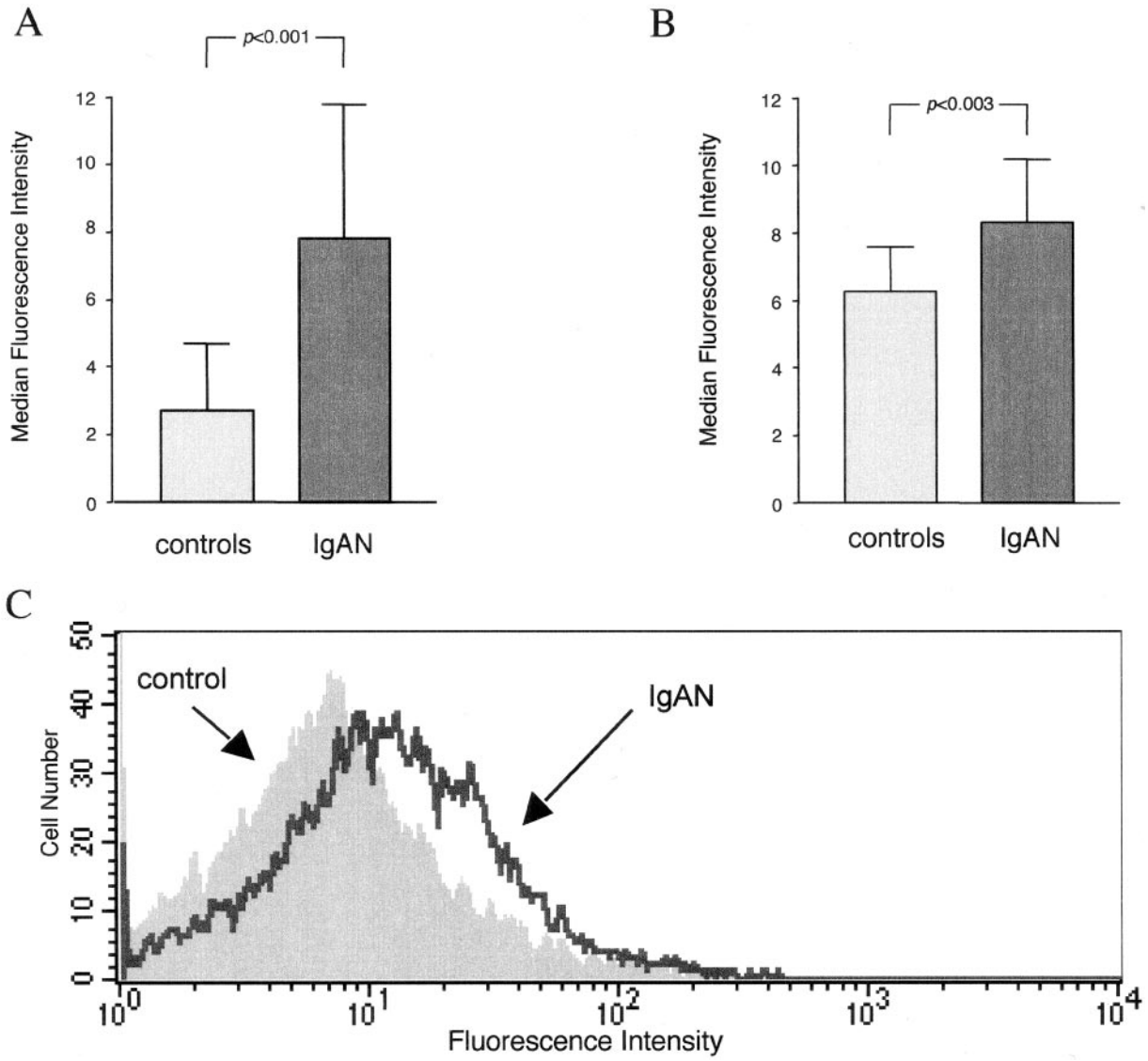

Figure 6. Enhanced binding of jacalin-purified IgA from IgAN sera to HMC and Daudi cells. (A) Comparative immunofluorescence analyses of binding of jacalin-purified IgA from 12 patients with IgAN and 12 control subjects (20 μg each) to HMC. (B) Binding of jacalin-purified IgA from patients with IgAN and control subjects to Daudi cells. Binding was evaluated by CH-EB6-8 mAb followed by streptavidin-APC. Median fluorescence intensities were plotted after subtracting values of each corresponding concentration of control IgG (mean ± SD). (C) Affinity-purified IgA1 from patients with IgAN bind more to HMC than controls. IgA1 from patients and control subjects were purified by affinity chromatography, and 20 μg of each was incubated with Daudi cells. IgA binding to Daudi cells was evaluated using biotinylated anti-human IgA mAb (CH-EB6-8) and streptavidin-APC as the developing reagent.

tion of the soluble TfR, we determined the levels of naturally occurring sTfR in serum. No significant differences were observed between control subjects and patients with IgAN (not shown). Therefore, these data indicate that IgA1 from patients with IgAN do not induce release of sTfR.

Discussion

Fc receptors are involved in the etiopathogenesis of many glomerulonephritides varying from autoimmune to those of systemic origin (*e.g.*, lupus nephritis, Goodpasture syndrome, IgAN) (23, 40). In IgAN, the identity of an IgA receptor mediating the mesangial IgA1 deposition and cellular activation has been enigmatic for many years (4). Indeed, although the interaction of IgA1 with HMC has been demonstrated by several groups (15, 17–19, 21), the identity of this putative mesangial IgA1 receptor has been elusive. Despite the presence of aberrantly glycosylated IgA1 in IgAN, the ASGP-R, one of the most important receptors responsible for catabolism of proteins with terminal Gal and GalNAc, is not expressed on mesangial cells (15, 20) and neither are other IgA receptors such as CD89 and pIgR (15, 19, 20). Recently, we characterized a novel mesangial IgA1 receptor, the TfR or CD71 (25). This receptor is the only IgA receptor so far identified at the membrane of HMC. Its importance is underscored by the fact

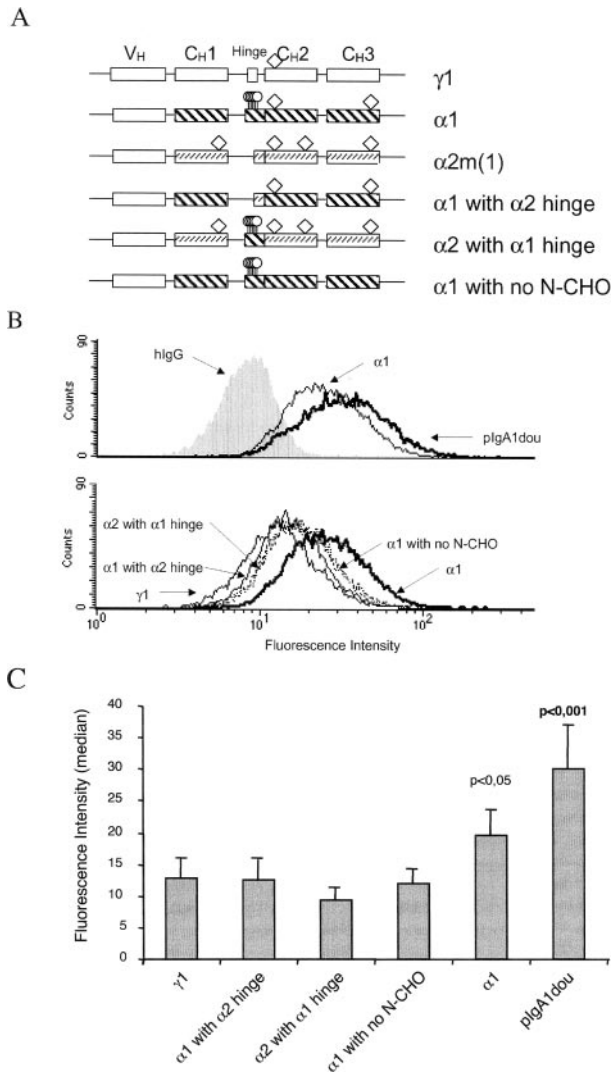

Figure 7. N- and *O*-glycosylation of IgA is important in IgA1/TfR interaction. (A) Schematic diagram of the H chain genes used to produce the recombinant antibodies. The exons are indicated by the rectangular boxes. Note that whereas the box between C_H1 and C_H2 codes for the of $\gamma1$ hinge region, the box at the 5' end of C_H2 in α codes for the hinge region. The open diamonds over the exons s show the position of *N*-linked glycosylation sites. The circles in the hinge of IgA1 represent the *O*-linked glycosylation sites. (B) Comparative immunofluorescence analyses of binding of different myeloma pIgA (pIgAdou), wild-type recombinant dimeric IgA1($\alpha1$), IgA1 with the hinge region of IgA2 ($\alpha1$ with $\alpha2$ hinge), IgA2 with the hinge region of IgA1 ($\alpha2$ with $\alpha1$ hinge), IgA1 lacking the *N*-linked glycosylation sites ($\alpha1$ with no N-CHO), and IgG1 have been previously described on Daudi cells. Cells preincubated with human IgG to mask FcγR were incubated with different IgA, and binding was evaluated by CH-EB6-8 anti-IgA mAb followed by streptavidin-APC. Human IgG (hIgG) was used as a negative control of binding (dashed line). (C) Median fluorescence intensities were plotted after subtracting values of each corresponding concentration of control IgG (mean ± SD; $n = 3$).

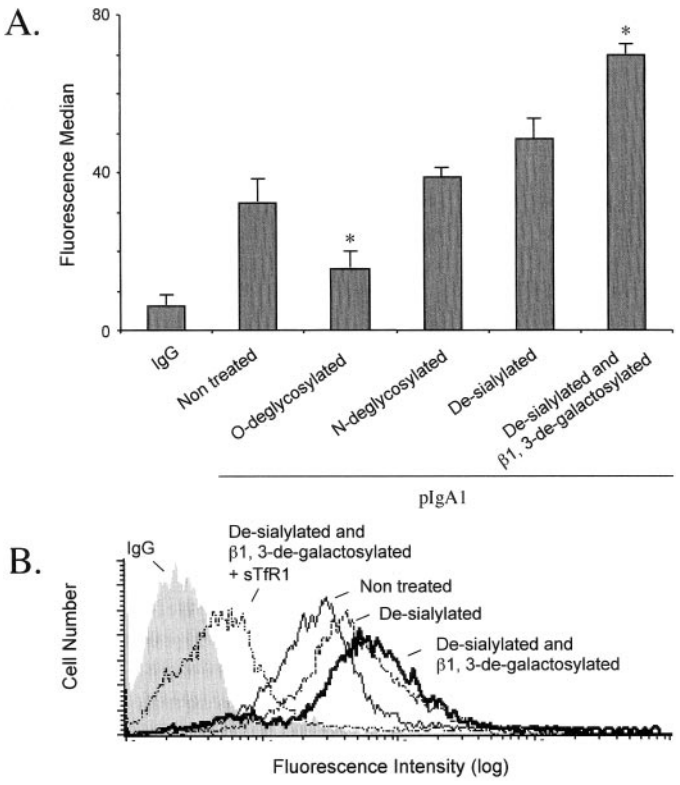

Figure 8. Desialylation and degalactosylation of IgA1 enhances its binding capacity to TfR. (A) Median fluorescence intensities were plotted (mean ± SD; $n = 3$) of binding of myeloma pIgA1 and of desialylated or deglycosylated IgA1 on Daudi cells as indicated. IgG was used as a negative control. Cells preincubated with human IgG to mask FcγR were incubated with different IgA, and binding was evaluated by CH-EB6-8 anti-IgA mAb followed by streptavidin-APC; $*P < 0.05$. (B) Comparative immunofluorescence analyses of binding properties of myeloma pIgA1 and of desialylated and degalactosylated IgA1 on HMC as indicated. The interaction to TfR was evaluated by using sTfR1 as a specific inhibitor.

that in IgAN, TfR expression is enhanced in the mesangium and co-localizes with IgA deposits (25, 26).

In this study, we first analyzed the interaction between different molecular forms of IgA and TfR expressed on cultured HMC. Using an indirect immunofluorescence assay with an anti-IgA mAb, we showed that polymeric but not monomeric IgA could bind to TfR expressed on mesangial cells and to other cells that have IgA receptor expression restricted to TfR, such as the Daudi cell line (data not shown).

Furthermore, we showed that IgA1 but not IgA2 binds the TfR on HMC and Daudi cells. However, these results showing no IgA2 binding to HMC are in contrast with previous observations using direct measurements of radiolabeled IgA2 binding (15). This discrepancy could be due to a higher sensitivity of the latter assay than the indirect immunofluorescence technique used here. The observations that mesangial IgA deposits in IgAN are mainly composed of IgA1 polymers and that TfR is overexpressed in the mesangium of patients with IgAN and co-localizes with IgA1 deposits (26) suggest that the TfR may be involved in binding of pIgA1 to HMC.

The specificity of IgA1-TfR interaction was confirmed by the inhibition of IgA binding to HMC by recombinant TfR soluble forms. As both TfR1 and TfR2 ectodomains could

block IgA1 binding to HMC, we postulate that the TfR domain involved in IgA binding is shared between these two receptors. This could be explained by the significant homology (45% amino acid identity) observed between these proteins (41). TfR2 differs from TfR1 only by a shorter side chain encoded by a separated gene (42), and TfR2 was shown to bind transferrin with low affinity and to mediate iron uptake (43). Our confocal data indicate that pIgA1 can induce TfR endocytosis in HMC with accumulation in intracellular vesicles from 15 to 60 min of endocytosis. One can propose that recycling of TfR can amplify mesangial IgA complex deposition, thus favoring subsequent activatory mechanisms that could lead to mesangial activation in IgAN (43). The intracellular localization of IgA in TfR-positive vesicles suggests that both molecules are targeted together to endolysosomal vesicles where pIgA are degraded. Whether both types of TfR are expressed by HMC and could play a role in IgA1-mediated signaling and/or endocytosis remains to be determined.

IgA1 mesangial deposits have been reported to derive from CIC (7). The data presented here reveal an increased capability for macromolecular IgA from patients with IgAN to interact with the TfR expressed on HMC. These findings point to a role of the molecular size of IgA in the interaction between CIC and mesangial TfR, refining observations recently reported by others (15). The specificity of macromolecular IgA/TfR interaction on HMC was also shown by using soluble forms of TfR1 and TfR2 as inhibitors. Both soluble forms of TfR induced approximately 50% inhibition of IgA binding to cell membrane. The enhancement of IgA1 binding to TfR only when IgA is present in IgA-complex form indicates that IgA1 interaction with the TfR is of a very low affinity and that its binding can be modulated by increased avidity after polymerization or aggregation. The failure to block completely IgA binding to HMC could be due to a lower affinity/avidity of soluble *versus* membrane TfR or could indicate that a second type of as-yet-uncharacterized mesangial IgA receptor may exist. A candidate molecule for such a second mesangial IgA receptor is the recently cloned Fcα/μ receptor, a new Fc receptor for IgA and IgM (44). Indeed, recently, transcripts of Fcα/μ receptor were described in mesangial cells that were upregulated after stimulation by proinflammatory cytokines such as IL-1β and TNF-α (45). Fcα/μ receptor contains only one Ig-like domain that is homologous to the first Ig-like domain of pIgR involved in IgA/IgM binding. However, this receptor does not seem to account for IgA binding in quiescent HMC because IgM cannot inhibit IgA binding to HMC (21). Furthermore, we produced recombinant ectodomain of Fcα/μ receptor in baculovirus/insect cell system, and this protein failed to inhibit IgA1 binding to HMC (data not shown). Although IgA interaction with the human Fcα/μ receptor on mesangial cells has not yet been demonstrated, a putative role of Fcα/μ receptor cannot be excluded during inflammation.

Several authors have postulated that aberrant glycosylation of the hinge region of IgA1 is involved in formation of immune complexes or autoaggregation that may promote formation of mesangial IgA1 deposits (7). Aberrant patterns of glycans, such as increased exposure of GalNAc residues and decreased content of Gal, have been detected in CIC as well as in mesangial deposits from patients with IgAN (46). The presence of this Gal-deficient termini in the hinge region of IgA1 could increase the exposure of antigenic determinants, which would be recognized by naturally occurring antibodies with anti-glycan specificity, inducing the formation of CIC (8). In the present work, we demonstrated the role of aberrant glycosylation in favoring the binding of Gal-deficient IgA and IgA–CIC to TfR on mesangial cells. Our data showed that IgA purified from sera of patients with IgAN has an increased ability to bind TfR expressed on HMC and on the Daudi cell line (that expresses TfR as the only IgA receptor) (25). Together with our present observation that there is no TfR interaction with a recombinant dimeric IgA1 containing the IgA2 hinge region, these data reinforce and explain our previous observation that IgA1 but not IgA2 can bind to TfR ((25) and data presented here). Our data indicate that the binding site of IgA1 on TfR involves the IgA1 hinge region. However, IgA1 hinge region alone is not sufficient to promote binding to TfR as evidenced with an IgA2 mutant containing IgA1 hinge region. In addition, other IgA domains could play a role because IgA1 without *N*-linked carbohydrates failed to bind the TfR. Taken together, one can conclude that IgA1 glycosylation influences IgA1 interaction with the TfR. By analogy with other Ig, one could speculate that sugars may be of importance for a correct folding of IgA1 (47). In addition, we still cannot exclude that some of these sugars may actually participate in the binding site itself, whereas other sugars may influence the binding indirectly by altering the IgA conformation. However, our experiments with deglycosylated IgA1 strongly point to a major role of *O*-linked rather than *N*-linked sugars in the interaction with the TfR. It is interesting that desialylation and degalactosylation of IgA1 induces a two- to threefold increase in binding to TfR as observed in Daudi cells and because binding of these IgA1 to HMC was strongly inhibited by the sTfR1. This may provide a link to TfR for previous observations that IgA1 molecules containing Gal-deficient *O*-linked glycans and CIC containing aberrantly glycosylated IgA have an enhanced binding to HMC (15), yet in IgAN, that IgA1 is found deposited in the mesangium and that IgA1 demonstrate an altered glycosylation are consistent with our observation that IgA1 binding to the TfR (the only IgA receptor identified so far on HMC) is modulated by the glycosylation state of IgA1. Indeed, IgA1 eluted from kidney are hypogalactosylated (46, 48), providing further evidence that hypogalactosylation of IgA1 promotes IgA1 mesangial deposition through the TfR.

Together, these data are consistent with the hypothesis on the role of aberrant IgA1 glycosylation in IgAN (49) and may be explained by the influence of aberrant glycosylation on TfR/IgA1 interaction. Furthermore, this is the first piece of evidence showing that altered glycosylation of the hinge region of IgA1 in patients with IgAN has a direct relationship with the increased interaction of IgA1 with the mesangial TfR. Whether altered glycosylation of IgA1 is the initial event resulting in increased TfR expression in the mesangium of patients with IgAN remains to be addressed. Finally, the absence of increase in soluble TfR levels in serum from patients suggests that

increased IgA1 binding to HMC in IgAN does not induce a shedding process with the release of soluble TfR in the serum.

In conclusion, we identified for the first time that two factors influence IgA1 interaction with the TfR, namely their molecular size and glycosylation. The inhibition of IgA1 binding by soluble TfR molecules could open avenues as a complementary approach in future therapeutic strategies to be used in IgAN.

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