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The IgA/IgM Receptor Expressed on a Murine B Cell Lymphoma Is Poly-Ig Receptor

Julia M. Phillips-Quagliata,* Samir Patel,* Jing-Kang Han,* Sergei Arakelov,* T. Dharma Rao,* Marc J. Shulman,† Shafie Fazel,† Ronald B. Corley,‡ Margaret Everett,§ Michel H. Klein,* Brian J. Underdown,‖ and Blaise Corthesy

T560, a mouse B lymphoma that originated in gut-associated lymphoid tissue, expresses receptors that bind dimeric IgA and IgM in a mutually inhibitory manner but have little affinity for monomeric IgA. Evidence presented in this paper indicates that the receptor is poly-Ig receptor (pIgR) known in humans and domestic cattle to bind both IgA and IgM. The evidence includes the demonstration that binding of IgM is J chain dependent, and that pIg-precipitated receptor has an appropriate *M, of 116–120 kDa and can be detected on immunoblots with specific rabbit anti-mouse pIgR. Overlapping RT-PCR performed using template mRNA from T560 cells and oligonucleotide primer pairs designed from the published sequence of mouse liver pIgR indicate that T560 cells express mRNA virtually identical with that of the epithelial cell pIgR throughout its external, transmembrane, and intracytoplasmic coding regions. Studies using mutant IgAs suggest that the Ca2 domain of dimeric IgA is not involved in high-affinity binding to the T560 pIgR. Inasmuch as this mouse B cell pIgR binds IgM better than IgA, it is similar to human pIgR and differs from rat, mouse, and rabbit epithelial cell pIgRs that bind IgA but not IgM. Possible explanations for this difference are discussed. All clones of T560 contain some cells that spontaneously secrete both IgG2a and IgA, but all of the IgA recoverable from the medium and from cell lysates is monomeric; it cannot be converted to secretory IgA by T560 cells.

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Materials and Methods

Cell lines

The T560.2.F7 and CH12.LX B lymphoma and the J774 macrophage cell lines were maintained as previously described (9, 22).

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Antibodies

All goat Abs and Ab conjugates were obtained from Southern Biotechnol-

ogy Associates (Birmingham, AL). Rat Abs to the following four mouse
C chains, 33-24, rat IgG2a anti-C\textsubscript{\textalpha}; 7/67, rat IgG1 anti-C\textsubscript{\textbeta}; IM4.1,
rat IgG1 anti-C\textsubscript{\textgamma}; and C2-23, rat IgG2a anti-C\textsubscript{\textdelta} were kindly pro-
vided by Dr. Richard Lynch (Department of Pathology, University of Iowa
College of Medicine, Iowa City, IA). Rabbit anti-recombinant mouse pIgR
reactive with denatured pIgR and SC in Western blots has been described
previously (23). The mouse anti-\kappa chain mAb RB7.9.1 (24) was puri-
fied from culture supernatants on protein A beads.

Detection of IgA and IgM binding by flow cytometry

Binding of IgA and IgM for detection by flow cytometry was done as
described in Ref.9 except that binding was at room temperature rather than
37°C. Various concentrations of IgA, IgM, or IgA-2,4,6 trinitrophenyl
(TNP)-BSA IgA were used, or for ELISA assays, mixtures of IgA, IgM, and
IgG, or anti-IgM Ab was used. Maximal binding of IgA or IgM was observed on TS60 cells passed 24 hours after exposure to 2 × 10^5 cells/ml,
incubated at 37°C for 2 h in fresh medium, then washed three times with Dulbecco’s PBS (DPBS) at room temperature followed by buffer appropriate to the assay. Binding of mouse IgM was detected with FITC-goat anti-mouse \kappa chain or, when only MOPC-104E
IgA was used, with FITC-goat anti-mouse \lambda chain; that of rat IgM was
detected with FITC-goat anti-rat IgM, that of mouse IgA was detected with
FITC-goat anti-mouse \alpha chain, and that of human IgA was detected with
FITC-goat anti-human \alpha chain.

For inhibition studies, the percentage of inhibition was determined from the
mean fluorescence (MF) in the various samples using the following

Immunoglobulins

**Human IgA.** Polymeric myeloma IgAA (IgA polymer 1) and monomeric
myeloma IgAA from a single individual (Steele) were purified in the lab-
oration of one of us (B.J.U.). Polymyeloma myeloma IgAA (IgA polymer 2)
and dimeric myeloma IgAA from two different individuals, McElhaney and
Latimer, respectively, as well as normal human S-IgA, were kindly given to
us by Dr. J. Mestecky (University of Alabama, Birmingham, AL).

**Mouse IgA.** BALB/c mouse myeloma IgAs, TEPC-15 (T15; IgA) and
MOPC-315 (M315; IgAA) were prepared as previously described (9, 21) or
purchased from Sigma-Aldrich. Mutant M315 IgA myeloma proteins were
prepared by cassette mutagenesis, changing Cys\textsubscript{575} to Ala, Tyr\textsubscript{302} to Phe,
and Cys\textsubscript{414} to Ala, and substituting the flexible human IgA1 hinge or the
very short human IgA2 hinge for the mouse hinge region. Briefly, the
pSV2-V\textsubscript{15} 315 plasmid was constructed by inserting an XbaI-NaeI fragment of
J\textalpha-14-V\textsubscript{30} and together with an enhancer-containing Novel
EcoRI fragment of a JH3-JH4 probe into the Xbal and EcoRI sites of the
multiple cloning site in pSV2NeoM (25). The wild-type (wt) pSV2-V\textsubscript{15} - C\textalpha
plasmid was created by inserting a 1.5-kb XhoI-Sall fragment containing the
BALB/c germline C\textalpha gene derived from the C3c0 phase clone (26)
into the corresponding site of pSV2-V\textsubscript{15} 315. PUC-C\textalpha 30 was made by excising the novel EcoRI fragment from pSV2-V\textsubscript{15} - C\textalpha
and cloning it into a modified pUC18 with no EcoRI and one XhoI site. IgA
mutant constructs were generated from pUC-C\textalpha 30. The region encompass-
ing residues 297–314 of wt C\textalpha 30 is flanked by EcoRI and EcoR47H11
restriction sites, which facilitated insertion of synthetic oligonucleotides
containing the desired mutations. The mutants were sequenced to ensure a
correct reading frame, and the mutant C\textalpha was excised from pUC with
XhoI-Sall and inserted into the pSV2-V\textsubscript{15} 315 plasmid. The wt and mutant
IgA constructs were electroporated into cells of the light-chain-expressing
recipient myeloma line M315.26. Mutant and wt IgAs in the supernatants
of the transfected cells were used to coat TNP-ox RBC (TNP-ORBc) for
flow cytometry (9). ELISA and agglutination titers were used to adjust
the IgA concentrations to approximate those in our standard preparation of
M315 IgA myeloma protein.

**Mouse IgM.** MOPC-104E (IgMA) and TEPC-183 (IgMA) were obtained
from Sigma-Aldrich. TNP-specific normal and mutant IgMs were prepared from
the supernatants of transfected cell lines expressing a TNP-specific
\kappa chain together with a normal or mutant TNP-specific \mu chain as previ-
ously described (27–29). SP-6 IgM has a wt \mu chain and consists mainly of
polymers with J chain; J/IgM-S414 and J/IgM-S575 both have \mu chains with Ser substituted for Cys\textsubscript{414}, but J/IgM-S575 consists mostly of
pentamers with some pentamers and tetramers lacking J chain, whereas
J/IgM-S414 (28) consists of polymers with J chain. J/IgM-S575 has
\mu chains with Ser substituted for Cys\textsubscript{575} and consists mainly of monomers
with some polymers lacking J chain. These IgMs were isolated from the

supernatants by binding to DNP-Sepharose followed by elution with 8 mM
DNP-glycine. The eluates were dialyzed against PBS to remove the hapten
and concentrated by ultrafiltration. A total of 200 \mu l of the concentrated
SP-6, J/IgM-S414, and J/IgM-S575 protein solutions were ultracentri-
 fuged on sucrose density gradients, essentially as described (27). Fractions
(0.5 ml) collected from the top down were serially diluted and examined by
ELISA, and the concentrations of IgM were plotted (Fig. 1). Pools of
“monomer” (fractions 4–8) and “polymer” (fractions 12–20) were concen-
trated by ultrafiltration and dialyzed against DPBS to remove sucrose, and
their IgM concentrations were determined by ELISA. Insufficient SP-6
monomer was obtained for experiments so only the polymer was used. The
J/IgM-S414 was concentrated but not subjected to sucrose gradient frac-
tionation. Mouse IgM was also isolated from supernatants of WEHI-231,
which lacks J chain and makes mainly hexameric IgM, and from two
WEHI lines that were transfected with the J chain gene as previously de-
scribed (30). Of these two cell lines, one, 3-H1, remains J chain negative;
the other, 3-C10, is J chain positive. The IgMs were isolated by binding to
Con A beads in the presence of 1 mM CaCl\textsubscript{2}, MgCl\textsubscript{2}, and MnCl\textsubscript{2} and, after
washing the beads, eluting the IgM with 0.2 M methyl-\alpha-\(-m\)annopyrano-
side (Sigma-Aldrich). The eluates were concentrated by ultrafiltration, di-
alyzed against DPBS, and the IgM concentrations were determined by
ELISA (see below) and adjusted to working concentrations by dilution in
DPBS.

**Other IgS**

Mouse IgG2ax (UPC-10), IgG2bx (MOPC-141; Sigma-Aldrich), and rat
IgM (Rockland, Gilbertsville, PA) were taken to have the concentrations
specified by the manufacturer. Normal Sprague Dawley rat-IgG was pre-
pared to be binding to and elution from protein G beads (Pierce, Rockford,
IL). Its concentration was determined from its OD\textsubscript{280}.

**Other proteins**

Mouse whey was kindly given to us by Dr. Michael Lamm (Department of
Pathology, Case Western Reserve University, Cleveland, OH).

Phosphatidylinositol-specific phospholipase C (PI-PLC)

treatment of T560.2F7 cells

T560.2F7 cells were incubated at 37°C with 50 \mu M PI-PLC from Bacill-
tus cereus (Boehringer Mannheim, Indianapolis, IN) and from Bacillus
thuringiensis (ICN Pharmaceuticals, Costa Mesa, CA) in medium or in
medium alone for 45 min in the presence or absence of 0.2 ng/ml calphos-
tin C (Calbiochem-Novabiochem, La Jolla, CA), washed three times in
medium containing FCS, and resuspended in DPBS for rosette assay as
previously described (9).

ELISA

ELISA were performed as described in Ref. 21 using plates sensitized with
gold anti-mouse \mu chain or anti-mouse or -human \alpha chain in PBS (pH 7.0).
The concentrations of the various mouse IgM and IgA and human pIgA
preparations were determined by interpolation on standard curves. Because
polymeric IgM and IgA titrate in ELISAs with different slopes from their
monomeric counterparts, both the samples and the standards, MOPC-104E
for mouse IgM, M315 for mouse IgA (both from Sigma-Aldrich), were
reduced to monomers and alkylated before titration. Briefly, 0.1-ml vol-
umes of IgM or IgA in PBS containing 1 mg/ml BSA were mixed with 0.3

FIGURE 1. Sucrose gradient separation of mutant IgMs J/IgM-S414, J/IgM-S575, and wt SP-6 IgM. IgM fractions 4–8 and 12–20 were pooled as “monomer” and “polymer,” respectively.
ml 0.1 M Tris-HCl buffer (pH 8.6). DTT (0.1 ml, 0.03 M in the same buffer) was added, and the mixtures were incubated for 1 h at 37°C. After reduction, iodoacetamide (0.1 ml, 0.07 M in buffer) was added, and the mixtures were incubated for 20 min at 37°C. Sodium acetate buffer (0.4 ml, 0.1 M) was then added to bring the pH to ~7.0 and the volume to 1.0 ml. Serial dilutions of the standards and samples were applied to the sensitized ELISA wells. The human IgaAs had titration curves similar to that of the standard (human colostral IgA from Sigma-Aldrich) and so were not reduced, but the monomeric human IgaA preparation had a titration curve of very different slope so its concentration was calculated simply from its OD. The concentration of rat IgM (Rockland) was taken as that specified by the manufacturer.

To check that the ability of the rat anti-mouse C\textsubscript{\mu} domain mAbs to bind to mouse IgM was intact, ELISA wells coated with 10 μg/ml of MOPC-104E IgM were blocked with BSA, and serial dilutions of the mAbs or of normal rat IgG were added to the wells and incubated. After washing, rat IgG bound to the wells was detected with HRP-goat anti-rat IgG Ab. All of the mAbs bound strongly to the IgM-coated wells and binding was not diminished at all by serial dilution down to 1.5 μg/ml. At this concentration, binding of normal rat IgG was at background levels.

### Preparation of Iga IC in Ab excess

To assay the ability of IgM to inhibit binding of mouse IgaA to the Iga/IgM receptor by flow cytometry, the Iga signal was increased by using IgA IC prepared in such vast Ab excess that no precipitation of 125 I-labeled IgA/IgM receptor from the surface of T560.2.F7 or CH12.LX cells resuspended in 0.4 ml DPBS were added 20 μl DPBS containing 4 U lactoperoxidase (Sigma-Aldrich) followed by 1.5–2 μl carrier-free \textsuperscript{125}I as NaI (ICN Biomedicals, Irvine, CA). Ten microliters 0.05% hydrogen peroxide in H\textsubscript{2}O were then added five times over the next 30 min, and the mixture was allowed to stand 10 min after the last addition. The volume was then brought to 10 ml with DPBS, and the cells were spun down and washed four times with DPBS. Aliquots of 2 × 10\textsuperscript{12} I\textsuperscript{25}I-labeled cells in 0.2 ml DPBS were lysed with 1 ml DPBS-based lysis buffer (pH 6.0) containing 0.5% Nonidet P-40, 0.01% soybean trypsin inhibitor, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml chymostatin, 2 μg/ml antipain, 100 μg/ml PMSF, 10 mM benzoamide hydrochloride, 50 mM e-aminocaproic acid, 20 mM iodoacetamide, and 0.02% sodium azide (32) at 4°C for 1 h. After centrifugation, the supernatants were transferred to clean tubes and supplemented with 1/10 volume of 3.5 M NaCl containing 100 μg/ml PMSF and 3 mg/ml protease-free BSA. The supernatants were precleared by rotating them at 4°C overnight with mixtures of 30 μl each of packed plain protein G beads, protein G beads coated with goat anti-mouse IgM (for CH12 cells), IgA, and IgG and protein G beads coated with the eventual Ab to be used to bind Iga IC or IgG to the beads to precipitate the receptor (i.e., goat anti-mouse \lambda light chain for MOPC-104E or M315, and anti-rat \kappa-chain mAb RG79.11 (24) for rat IgM). These beads were removed by centrifugation, and a second preclearance was performed by transferring the supernatants into fresh tubes containing 30 μl of packed MOPC-21 (mouse IgG1)-coated beads, rotating the mixtures at 4°C for 2 h. Equal volumes of the supernatants were then placed into fresh microfuge tubes containing 30 μl washed protein G beads precoated with either monoclonal anti-rat \kappa-chain followed by rat IgM or control, BSA-containing buffer, or goat anti-mouse \lambda light chain Ab followed by either M315 dimeric IgA, MOPC-104E IgM, or control, BSA-containing buffer. The lysates were rotated with the beads overnight at 4°C. The beads were recovered by centrifugation and washed twice with 1 ml high-salt lysis buffer, five times with 0.5 ml of this same buffer, and once with 1 ml low-salt lysis buffer. The protein was solubilized from the beads by boiling with 50 μl fresh Laemmli sample buffer (33) with or without 0.7 M 2-ME and electrophoresed on SDS-PAGE. In some experiments, precipitation was done in ELISA plates instead of on beads (32). For preclearance, the wells were coated with goat anti-mouse \lambda light chain or TNP-BSA, washed with PBS, and blocked with BSA. The lysates were precleared seven times by serial transfer to and incubation at 4°C in such coated wells, then placed in wells coated with goat anti-mouse \lambda light chain, followed by either MOPC-104E IgM, control BSA-containing buffer, or with TNP-BSA followed by M315 dimeric IgA or BSA-containing buffer. After adsorption to the coated wells, the lysates were removed, and the wells were washed seven times with PBS. Bound proteins were removed from the wells with Laemmli sample buffer with or without 0.7 M 2-ME and boiled for 5 min before separation on SDS-PAGE.

### Immunoblotting

For immunoblotting, lysates or 10-fold concentrated supernatants of unlabeled cells were precleared with protein G beads precoated with goat anti-mouse \kappa\textsubscript{\lambda} chain (T560 cells) or goat anti-mouse \kappa\textsubscript{\mu} chain for MOPC-104E or M315, and anti-rat \kappa-chain mAb RG79.1. For immunoblotting, lysates or 10-fold concentrated supernatants of unlabeled cells were precleared with protein G beads precoated with goat anti-mouse \kappa\textsubscript{\lambda} chain (T560 cells) or goat anti-mouse \kappa\textsubscript{\mu} chain for MOPC-104E or M315, and anti-rat \kappa-chain mAb RG79.1. For immunoblotting, lysates or 10-fold concentrated supernatants of unlabeled cells were precleared with protein G beads precoated with goat anti-mouse \kappa\textsubscript{\lambda} chain (T560 cells) or goat anti-mouse \kappa\textsubscript{\mu} chain for MOPC-104E or M315, and anti-rat \kappa-chain mAb RG79.1. For immunoblotting, lysates or 10-fold concentrated supernatants of unlabeled cells were precleared with protein G beads precoated with goat anti-mouse \kappa\textsubscript{\lambda} chain (T560 cells) or goat anti-mouse \kappa\textsubscript{\mu} chain for MOPC-104E or M315, and anti-rat \kappa-chain mAb RG79.1.

### Table I. PCR primers used for sequencing the pIgR of T560

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BSA (controls), or with goat anti-\(\alpha\)-chain Ab. For the plgR, 10% SDS-PAGE was used, and the proteins were electrophoretically transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). For S-IgA, 5% SDS-PAGE was used, and the proteins were transferred to \(\zeta\)-probe (Bio-Rad).

The membranes were blocked with 10% BSA in PBS containing 0.05% Tween 20 and 0.02% sodium azide, washed in PBS-Tween 20 without azide, and stained with either rabbit anti-denatured recombinant plgR (23) or normal rabbit serum followed by HRP-conjugated goat anti-rabbit IgG heavy and light chains (human/mouse adsorbed) or HRP-conjugated goat anti-mouse \(\alpha\)-chain, diluted in 5% normal goat serum/PBS-Tween 20. The bands were developed with ECL Western blotting detection reagents (Amersham, Arlington Heights, IL).

**Results**

**Cross-inhibition of binding of IgA and IgM to T560.2.F7 cells**

Rosette assays were previously used to show that T560 cells bind both mouse IgA- and IgM-coated erythrocytes and that these two Igs are mutually inhibitory (9). Similar binding and inhibitory phenomena are here demonstrated by flow cytometry with both mouse and human IgA and mouse and rat IgM. Binding of polymeric M315 mouse IgA myeloma protein to T560.2.F7 cells in the presence or absence of TNP-BSA is shown in Fig. 2A. IgA binds to T560.2.F7 cells, and the presence of TNP-BSA in Ab excess markedly enhances the signal from FITC anti-\(\alpha\)-chain Ab, presumably because there is more IgA to take it up in the bound complex. Not all of this IgA is necessarily in contact with receptors on the cell surface, but multipoint attachment to the cell surface receptors probably increases the avidity of binding of complexes over that of plgR alone. No binding to CH12.LX cells, which lack IgA/IgM receptors, is seen (Fig. 2B).

Inhibition of binding of M315 IgA-TNP-BSA IC to T560.2.F7 cells by normal rat IgM is illustrated in Fig. 3. More than 50% inhibition of binding of IgA complex containing 25 \(\mu\)g/ml IgA is achieved with as little as 9 \(\mu\)g/ml of rat IgM.

Binding of 40 \(\mu\)g/ml of MOPC-104E mouse IgM to T560.2.F7 cells is inhibited by human polymeric and dimeric IgA, but not at all by S-IgA and only very little by monomeric IgA. Fifty percent inhibition requires \(\sim 100 \mu\)g/ml of IgA polymer (1) and dimer, but 350 \(\mu\)g/ml of IgA polymer (2) and \(>1000 \mu\)g/ml IgA monomer (Fig. 4). Because the inhibition curves for polymer and monomer have the same slope, it is possible that the inhibition by monomer is due to minor contamination by polymer.

We previously noted (9) that high concentrations of both mouse IgG2a (UPC-10) and IgG2b (MOPC-141) inhibited IgA rosetting formation. These two proteins were shown not to be appreciably contaminated with IgA. In the present work, we tested the same lots of protein for their ability to inhibit binding of MOPC-104E IgM to T560.2.F7 cells as measured by flow cytometry. We found that IgG2a inhibited IgM binding by 40% at 300 \(\mu\)g/ml and by 16% at 150 \(\mu\)g/ml whereas, at the same concentrations, IgG2b enhanced it (data not shown). ELISA subsequently revealed that the IgG2b preparation was contaminated by IgM, which would
explain why it inhibited IgA rosette formation in our earlier experiments. However, the inhibition of both IgA and IgM-binding by IgG2a is still unexplained for it was not contaminated by either IgA or IgM. Inhibition of IgM binding by IgG2a in the experiments discussed here is much less impressive than the 100% inhibition of IgA rosette formation seen with only 65 μg/ml IgG2a in our earlier study (9).

Binding of IgA and IgM to the IgA/IgM receptor is not due to recognition of a shared carbohydrate

To examine the possibility that IgA and IgM bind to the IgA/IgM receptor through a shared carbohydrate, the effect of periodate oxidation of the carbohydrate on binding of rat IgM and M315 IgA was tested. The data (Table II) indicate that treatment with peridate has little or no effect on the ability of IgM to bind to the cells (in the first experiment it reduced it, in the second it did not) but, in three experiments, it increased the ability of IgA to do so. It is not clear why it has this effect on IgA but not IgM. One possibility is that cleavage of sugar rings in the carbohydrates on IgA renders the IgA susceptible to aggregation, another is that it relieves a major reduction in binding of either protein, it seems unlikely that interaction with the T560 receptor is mediated through a carbohydrate shared between IgM and IgA.

Binding of murine IgM to the T560 IgA/IgM receptor does not involve the same epitopes as binding to the IgM receptor on murine T cells

Marked inhibition of binding of murine IgM to the T cell IgM receptor was shown to occur when either of two different rat mAbs (IM41 and 2911) to the Cμ3 domain were complexed with the IgM at ratios higher than 5:1 (Ab to IgM) before addition to the cells (19). Borderline inhibition was seen with one of two mAbs to the Cμ2 domain and no inhibition was seen with anti-Cμ1 or with either of two mAbs to the Cμ4 domain. We tested the ability of some of these same Abs to inhibit binding of MOPC-104E to the T560 IgA/IgM receptor at Ab:IgM ratios ranging from 8:1 down to 1:1. There was no inhibition of binding with any of the mAbs to any of domains Cμ1 through Cμ4, most notably not with IM41, which reacts with Cμ3, indicating that the epitopes bound by the mAbs are not involved in binding to the T560 IgA/IgM receptor (data not shown). Marked potentiation of the signal provided by FITC-goat anti-mouse λ light chain actually occurred with Abs to Cμ2 and Cμ3 but not with Abs to Cμ1 and Cμ4, suggesting that the former promoted the build-up of MOPC-104E-Ab IC on the cell surface.

Role of J chain in the binding of IgM to the IgA/IgM receptor

The best described IgA/IgM receptor to date is the plgR, normally expressed on secretory epithelial cells. Expression of the structure recognized by this receptor is dependent on the presence of J chain in the IgA or IgM polymer (16–18). To examine a possible relationship between the T560 IgA/IgM receptor and the plgR, binding of IgMs containing or lacking J chain was investigated. TNP-specific SP-6 mutant and wt IgMs were affinity purified from the supernatants of transfected cells, separated into monomers and polymers on sucrose gradients (J+/IgM-S414, J−/IgM-S575, and wt SP-6) or left unseparated, (J+/IgM-S414), and their ability to bind to T560.2,F7 cells was assessed by flow cytometry (Fig. 5, A and B).

The data indicated that neither the polymers nor the monomers from the J− mutant IgMs could bind significantly to T560.2,F7 cells (there was a trace of binding of J+/IgM-S414 polymer) but J−/IgM-S414 and wt SP-6 polymeric IgM as well as additional control, unseparated IgMs, MOPC-104E, and TEPC-183 all bound very well. They suggested that plgM-lacking J chain does not bind to the receptor. However, they were not conclusive because the mutations in the μ-chains of S414 and S575 might have affected their ability to bind independently of affecting their J chain content.

The role of J chain in binding to the T560.2,F7 IgA/IgM receptor was further examined by comparing the binding of WEHI-231 IgM with normal μ-chains but lacking J chain (30) with that of IgM from two transfected cell lines: one, 3-C10, expressing J chain; the other, 3-H1, not expressing J chain. (Fig. 6). The results were clear cut. 3-C10 IgM-containing J chain, bound well to T560.2,F7 cells; WEHI-231 IgM and 3-H1 IgM, both lacking J chain, failed to bind. Because WEHI-231 IgM is substantially hexameric, the results support the conclusion that it is not sufficient for IgM to be polymeric; it must contain J chain to bind to the receptor. The results are consistent with the idea that the T560 IgA/IgM receptor is a form of plgR, known to require J chain for binding of polymeric Ig.

Precipitation with plg identifies a 116-kDa receptor for IgM/IgM on T560 cells

Radioiodinated IgA/IgM receptor was precipitated with plg from T560 cells under several sets of conditions. Both rat and mouse IgM precipitated it more readily than plgA. No similar molecule

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was precipitated from CH12 cells. In our earliest studies (20) using the protease inhibitors described in Ref. 14, triethanolamine, iodoacetamide, chymostatin, leupeptin, pepstatin, antipain, and PMSF, we precipitated small amounts of a molecule similar in size (36 kDa) to that of the IgA-binding 38-kDa molecule precipitated from murine T cells (35). However, when we added soybean trypsin inhibitor, aprotinin, diisopropylfluorophosphate, and sodium azide to the inhibitors mentioned above (32), we brought down a band of much higher $M_r$ (116 kDa; Fig. 7), and the 36-kDa band disappeared. It seems likely that the 36-kDa band represented an IgM-binding product of proteolysis derived from the 116-kDa band. The apparent $M_r$ of the 116-kDa band increased to about 120 kDa on 7.5% polyacrylamide gels but was unaltered under reducing vs nonreducing conditions (not shown). The band was also precipitated by IgA or IgM in PBS in the presence of EDTA indicating that its binding is not dependent on divalent cation (not shown).

Immunodetection reveals the identity of the 116-kDa protein as pIgR

The receptor precipitated from Nonidet P-40 lysates of T560 cells with either rat IgM or human plgA was detectable as a band of ~116 kDa on immunoblots with rabbit Ab to denatured mouse pIgR followed by HRP-goat Ab to rabbit IgG (Fig. 8). No such band was precipitated from lysates of CH12 cells, which lack the IgA/IgM receptor. Control lanes show both free SC (~90–95 kDa).
FIGURE 8. Immunoblot showing precipitation of 116-kDa band from T560 cells by rat IgM or human pIgA on protein G beads. The beads were preincubated with RG7.9.1 anti-rat κ-chain, washed, and then incubated with rat IgM (M), diluent alone (MC), goat anti-human λ light chain followed by human IgA polymer (1) (A), or diluent alone (AC). Reduced samples were run on 10% SDS-PAGE, blotted onto nitrocellulose, and developed by anti-pIgR Ab. Negative controls were done with CH12.LX cells stained with anti-pIgR (shown), as well as with T560 cells stained with normal rabbit serum followed by HRP goat anti-rabbit IgG (not shown). Nonreduced (NR) and reduced mouse whey provided positive controls for staining of intact S-IgA (the high Mr bands above 208 kDa) and free SC (the bands above 84 kDa) by the anti-pIgR Ab.

T560 cells express mRNA-encoding pIgR

The binding characteristics of the IgA/IgM receptor, especially its requirement for J chain, its high molecular mass, and its ability to bind anti-murine pIgR Ab suggest that it is pIgR. The possibility that it might represent some new B cell form of the pIgR, perhaps with GPI linkage to the cell membrane, as suggested by the sensitivity of the T560 IgA receptor to PI-PLC (9, 36), was next explored by RT-PCR. First we demonstrated (Fig. 9) that T560 cells contain message for the pIgR using primers encompassing ~80% of domain I and a small portion of domain II of the mouse liver pIgR sequence. Identically sized fragments (337 bp) were amplified from rat and mouse liver and from both of two separately prepared T560.2.F7 mRNAs but not from mRNA of either CH12.LX, the B cell line used as control above, or J774, a receptor-negative macrophage line (9), and not from a control containing no template. The fragments were TA-cloned and sequenced; fragments amplified from rat mRNA contained rat sequence while those amplified from mouse liver and from T560 contained mouse sequence.

Multiple, serial RT-PCRs were then performed using T560 mRNA as template and overlapping sets of primers. The fragments were TA-cloned and sequenced. The whole T560 pIgR sequence is shown in Fig. 10. T560 cells clearly contain mRNA coding for a protein identical with epithelial cell pIgR throughout its external, transmembrane, and intracytoplasmic regions except for a single T to C base change at position 476 that results in a Val→Ala change in domain II. This change, which could well be allelic, was verified by sequencing the product of an independent RT-PCR. Two C to T changes, one conservative in domain 4, the other in the 3’ untranslated region, have not been so verified. The sequence provided no grounds for supposing that the form of pIgR on T560 was different from that on epithelial cells.

The sensitivity of the pIgR on T560 cells to PI-PLC is due to activation of protein kinase C (PKC)

We previously noted (9) that treatment of T560 cells with PI-PLC destroyed their ability to bind IgA-coated erythrocytes, suggesting that the IgA receptor might be GPI-linked to the cell membrane. We also found that activation of protein kinase C (PKC) by PMA caused down-regulation of IgA receptor activity. Subsequently, we discovered (36) that the effect of PI-PLC was partially reversed by staurosporine, a protein kinase inhibitor. We now show that it is completely reversed by calphostin C, which is PKC-specific. As shown in Table III, treating T560 cells with PI-PLC reduces their ability to form IgA rosettes by ~85%. Addition of calphostin C diminishes the reduction due to PI-PLC to only 3%. These results suggest that PI-PLC causes loss of IgA receptor activity (by inference pIgR receptor activity) from T560 cells indirectly, by activating PKC. PKC activation may follow cleavage by PI-PLC of a bona fide GPI-linked molecule from the cell surface (or much less likely) by PI-PLC itself crossing the cell membrane and cleaving phosphatidylinositol bisphosphate. Whatever the mechanism of PI-PLC-induced activation of PKC, it is clear that the T560 pIgR is not GPI-linked to the cell membrane but has conventional type I transmembrane and cytoplasmic regions consistent with the sequence data presented above.

Lack of effect of mutation in the Ca2 domain or hinge regions on binding of IgA to the pIgR

Motifs in the Ca2 domain have been postulated to mediate binding between pIgA and the pIgR. To explore the roles of Ca2 domain Cys residues in the binding of IgA to the pIgR, rosette assays were performed comparing normal and mutant IgA proteins. The residues targeted were Cys301, its adjacent Tyr302, and Cys311. Both Cys301 and Cys311 are responsible for inter-α-chain bonding.
within the IgA monomer. In addition, Cys 311 of one α-chain in an IgA dimer undergoes a disulfide exchange reaction and binds to a highly conserved Cys in pIgR domain 5 (Cys 467 in human pIgR) during the formation of S-IgA. At similar concentrations and agglutination titers, there was no significant difference between mutant and nonmutant IgAs in their ability to mediate rosette formation (Table IV). Discrepancies between the concentrations and agglutination titers probably reflect the ratios of monomer to polymer in the different supernatants. Because either of two Cys and a Tyr in the Cα2 domain can undergo change without impairing the ability of the dimeric IgA molecule to bind to the pIgR, the Cα2 domain is probably not directly involved in high-affinity binding to the pIgR. It also makes no difference to rosette formation whether the extended, flexible human IgA1 or the very short human IgA2 hinge is substituted for the mouse hinge region.

The IgA secreted by T560 cells upon switching from IgG2a to IgA is all monomeric and is not taken up and resecreted as S-IgA. Because T560 cells can produce IgA and contain mRNA for J chain as determined by RT-PCR, we explored the possibility that T560 might make dimeric IgA that could bind to the pIgR and then be internalized, processed in endosomes, and released as S-IgA. In immunoblots (Fig. 11), the lanes containing the supernatants and lysates of T560.2.F7 contain α-chain bands with an Mr of ~100 kDa, consistent with α-chain dimers (the light chains of mouse IgA are not covalently bound to heavy chains). No tetrameric or higher molecular mass α-chain bands, such as are seen in the lanes containing T15 IgA, M315 IgA, and S-IgA (in mouse whey), are detectable.

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FIGURE 10. Comparison of the sequences of pIgR mRNA from mouse liver and from the T560 B lymphoma. Dashes indicate identity between the T560 and mouse liver sequences; horizontal arrows underline the sequences used to make the forward and reverse primers for RT-PCR; vertical lines define the domain borders; asterisks indicate the initiation and termination codons; and the CDR1, CDR2, and CDR3 homology regions of domain I are italicized.
Table III. Effect of calphostin C on PI-PLC-induced reduction in IgA rosette formation by T560.2.F7 cells incubated with M315 IgA-coated TNP-ORBC

<table>
<thead>
<tr>
<th>PI-PLC-1* (50 mU/ml)</th>
<th>PI-PLC-2 (50 mU/ml)</th>
<th>Calphostin C (0.2 ng/ml)</th>
<th>% IgA RFC a</th>
</tr>
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<tr>
<td>– – –</td>
<td>– – –</td>
<td>–</td>
<td>35.3</td>
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<tr>
<td>– – +</td>
<td>– – +</td>
<td>+</td>
<td>4.3 (87.8)</td>
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<td>– – +</td>
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<td>+</td>
<td>6.1 (82.7)</td>
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<td>+ – +</td>
<td>+</td>
<td>34.2 (3.1)</td>
</tr>
<tr>
<td>– + +</td>
<td>– + +</td>
<td>+</td>
<td>34.2 (3.1)</td>
</tr>
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</table>

* PI-PLC-1 was from B. cereus; PI-PLC-2 was from B. thuringiensis. A minimum of 200 cells per sample was counted in either the RFC or non-RFC category. Figures in parentheses represent the percentage decrement in RFC resulting from treatment. No RFC were found when T560 cells were incubated with control TNP-ORBC without M315 IgA.

Discussion

The IgA/IgM receptor expressed on the murine B lymphoma, T560, is clearly conventional pIgR, well known to function in the transport of dimeric or pIgA and IgM across epithelial cells into secretions. Binding to the T560 receptor by both IgA and IgM is independent of any hypothetical shared carbohydrate on these Igs, because periodate oxidation of the carbohydrates on the Igs does not prevent binding. This excludes the possibility that the T560 receptor band is related to the lectin-like IgD receptor because periodate oxidation of the carbohydrates on the Igs does not involve the Cα chain dependent as are binding of IgM and pIgA to the epithelial pIgR (16–18). Binding of IgM to the T560 IgA/IgM receptor does not involve the Cα3 epitope used in binding to the mouse T cell FcγR (19). IgA/IgM receptor precipitated from the T560 cell surface has an appropriately high M, (116 kDa), much higher than that of FcγR on either murine T cells (38 kDa; Refs. 14 and 35), human myeloid cells (30 kDa; Ref. 32), or human B cells (58 kDa; Ref. 40), or of the FcγR on human B cells (58–60 kDa; Refs. 41 and 42) or T cells (60 kDa; Ref. 42), and can be detected on immunoblots with rabbit Ab to murine pIgR. mRNA-encoding pIgR is expressed within T560 cells, and its sequence indicates that the molecule has external and transmembrane regions as well as a cytoplasmic tail identical with that of the molecule expressed on epithelial cells (34). Of course, because the sequence was determined from cloned RT-PCR-amplified products, it is possible that mRNA for a distinct, hypothetical B cell GPI-linked pIgR was also present, but not detected. This hypothesis is rendered unlikely by the following two findings: first, the loss of IgA receptor activity from the cell surface following PI-PLC treatment was shown to be due not to cleavage of a GPI-linked pIgR from the cell surface but rather to down-regulation of cell surface pIgR activity following activation of PKC; second, only one receptor band was detectable on immunoblots.

The ease with which wt rat or mouse IgM, as compared with dimeric or pIgA, binds to the pIgR on T560 cells is striking. Only 9 μg/ml rat IgM are needed for 50% inhibition of binding of MOPC-315 IgA IC containing 25 μg IgA/ml to T560 cells, whereas 100 μg/ml of polymeric or dimeric human IgA are needed for 50% inhibition of binding of IgM at 40 μg/ml. The affinity of the T560 pIgR for IgM vs IgA is difficult to compare with that measured in different laboratories because it depends on what species is under consideration and whether the pIgR is free (as SC in solution) or cell bound. For instance, human IgM has a Kd 2–12.5 times that of dimeric IgA in binding to human SC in solution (43, 44) but binds to human pIgR on the surface of transfected Madin-Darby canine kidney epithelial cells with much lower and more nearly equal affinity, though the amount of IgM bound per cell is 3-fold higher (45). The affinity of binding also depends on the source of the pIg; for example, one of us (R.B.C.) has found that while mouse pIgA, like human pIgA and IgM, binds to human SC in an ELISA, mouse IgM does not, implying that differences between human and mouse IgM affect their interaction with human SC; in addition, human pIgA has a lower affinity for human than for rabbit pIgR (46).

Although human pIgR and the T560 mouse pIgR bind both pIgA and IgM, rabbit (44) and rat (47) hepatocyte pIgR bind only pIgA well and do not translocate IgM into bile. Because mouse liver similarly translocates pIgA but not IgM into bile (48, 49), it is generally assumed that mouse hepatocyte pIgR resembles rat and rabbit pIgR and binds IgM poorly or not at all. If this is true, then the difference between the mouse hepatocyte and the T560 pIgR that makes the latter behave more like human pIgR must be explained. Given that the amino acid sequences of the mouse hepatocyte and T560 B cell pIgRs are the same except for the Val to Ala change in domain 2, the difference most likely reflects differential folding or glycosylation of the pIgR, probably the latter. It is easy to imagine that a bulky carbohydrate on hepatocyte-derived pIgR could interfere with IgM but not with IgA binding. Furthermore, it has already been shown that deglycosylation of human SC allows it to inhibit binding of biotinylated native SC to pIgA with 10 times greater efficiency than native SC itself (50), suggesting that some of the carbohydrate moieties on human pIgR may actually impede binding even of pIgA.

Initial, high-affinity, noncovalent binding of pIgA or IgM to the epithelial cell pIgR involves interaction with its first domain (50–54). This first domain of the pIgR contains three Ig-related...
blocking to T560 cells only when they contained J chain. However, it is still not known what function J chain performs in binding to plgR, and the requirement for it may not be absolute (16–18) because some plgAAs lacking J chain do bind to plgR (60, 61). J chain may merely hold the IgA and IgM subunits with their loops in a configuration necessary for their interaction with the plgR or may itself interact with plgR. Isolated J chain dimers bind only marginally to SC (16), but Abs to certain J chain epitopes can block binding of intact plg to SC (62) and prevent plgR-mediated biliary and epithelial transport of human plgA (63), suggesting that J chain may participate in binding to plgR. Only one J chain is present in any given IgA polymer or IgM pentamer (64). Differences in the sizes of the IgA polymers or ratios of IgM pentamer with J chain to hexamer without J chain in the proteins we used might account for the differences in binding observed in our experiments with human polymeric vs dimeric IgA and with MOPC-104E vs TEPC-183 vs the separated polymeric fraction of SP-6 IgM.

The ability of murine IgG2a (UPC-10) to inhibit binding of both plgA and IgM to the murine plgR is unexplained. T560 exhibits low-level direct binding of a second mouse IgG2a myeloma protein (UN2S1) in rosette assays (9) so the inhibitory effect may not be restricted to the UPC-10 IgG2a myeloma protein. Inhibition might depend on sharing of one or more binding structures between IgM, IgA, J chain or plgR, and murine IgG2a. Alternatively, it might depend on some interaction between IgG2a and another molecule on the T560 cell surface that down-regulates the plgR by, for example, activating PKC (9). Optimal expression of the plgR on T560 cells is seen when the cells have been cultured in fresh medium before being washed and used in the assay. This thorough washing procedure may allow the plgR on T560 cells to recover from down-regulation or inhibition by the IgG2a the cells themselves secrete. It was originally instituted because we thought that IgA secreted by T560 (20) might block the IgA/IgM receptor. However, this is unlikely to be the case because all of the IgA contained in both T560 cell lysates and supernatants (no more than 10 ng IgA/ml of supernatant at the end of a 3-day culture) is monomeric. Perhaps T560 cells do not make J chain due to some unknown defect in J chain mRNA (detectable in T560 by RT-PCR). None of the T560 IgA is converted to S-IgA, which is consistent with the fact that monomeric IgA does not bind to the plgR. We conclude that endogenously produced IgA is not capable of autocrine stimulation of T560 cells through the plgR.

Activation of PKC down-regulates T560 IgA binding activity (9, 35), which we now know represents plgR activity. The mechanism of down-regulation is not clear. We previously showed (9) that, after down-regulation by PMA, receptor activity recovered slowly (over several hours) even in the presence of cycloheximide, suggesting that the receptor had not been shed or degraded and that a recycling mechanism might be involved. In Madin-Darby canine kidney cells, activation of PKC by PMA causes transcytosis and apical recycling of transfected plgR but does not stimulate endocytosis at the basolateral surface; i.e., in these polarized cells, plgR is transcytosed from the basolateral surface to the apical recycling compartment and then delivered to the apical surface more rapidly in the presence than in the absence of PMA (65). T560 B cells are nonpolar, and nothing is known about the membrane compartment with which their plgR is associated. Perhaps activation of PKC in T560 cells promotes endocytosis of plgR rather than an increase in its surface expression.

That T560, a B lymphoma, expresses plgR at all is a novelty. It raises the question whether plgR expression occurs naturally on a small cohort of normal B cells, of which T560 is a transformed...
representative, or whether it is due to some aspect of the transformation process. Support for the notion that pIgR may be expressed on normal or activated lymphocytes comes from work showing that the splenocytes of mice carrying an IgA-secreting myeloma express IgA receptors whose ability to bind IgA is blocked by rabbit anti-rat SC (15). No other evidence indicating expression of pIgR by normal lymphoid cells has appeared, although numerous immunohistochemical studies have shown binding of anti-SC Abs to human intestinal epithelial cells in health and disease (65). If binding of anti-SC Abs to mucosal lymphocytes were common, it should surely have been recorded. Perhaps expression of the pIgR on the T560 lymphoma initially occurred as a response to cyto- kines available during malignant transformation. T560 is highly activated (22) with heavily mutated Ig heavy and light chains (21) and originated in the GALT of an F3 hybrid mouse that had been injected with parental splenocytes. Although signs of an ongoing graft-vs-host reaction were not apparent at the time of sacrifice, a graft-vs-host reaction had probably been initiated and then abro- gated. Graft-vs-host reactions involve release of many cytokines that might up-regulate pIgR on an activated B cell (64) that was subsequently transformed. In several human systems, synergy between IFN-γ and IL-4 in up-regulating pIgR has been reported (66). Whether these same cytokines would function in the same way with respect to murine B cells is unknown, but it may be significant that T560 secretes IL-4 (22). An alternative hypothesis, suggested to us by Dr. Randy Goldblum (University of Texas Medical Branch, Galveston, TX), is that the pIgR gene, normally localized to chromosome 1 in both human (67, 68) and mouse (69), has been switched on in T560 as a consequence of translocation events that put it under the control of the promoter of some other constitutively activated gene. We have not investigated the chromo- somes of T560 and do not know the genetic mechanism of its transformation.

References


