A Cell Surface Glycoprotein of Rat Basophilic Leukemia Cells Close to the High Affinity IgE Receptor (FcεRI)

SIMILARITY TO HUMAN MELANOMA DIFFERENTIATION ANTIGEN ME491

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A monoclonal antibody (mAb), AD1, was isolated that recognized a cell surface protein on rat basophilic leukemia cells (RBL-2H3). At high concentration, this antibody inhibited IgE-mediated but not calcium ionophore-induced histamine release (49% inhibition at 100 μg/ml). The mAb AD1 did not inhibit the binding of IgE or of several antibodies directed to the high affinity IgE receptor (FcεRI). Likewise, IgE did not inhibit mAb AD1 binding. However, several anti-FεRI antibodies did inhibit mAb AD1 binding as intact molecules but not as Fab fragments. Therefore, the sites on the cell surface to which mAb AD1 binds are close to FcεRI. The mAb AD1 immunoprecipitated a broad, 50-60-kDa band from 125I-surface-labeled RBL-2H3 cells that upon peptide N-glycosidase F treatment was transformed into a sharp 27-kDa band. A similar 27-kDa protein was immunoprecipitated from surface-radiolabeled cells after culture with tunicamycin. Thus, the protein recognized by mAb AD1 is highly glycosylated with predominantly N-linked oligosaccharides. The N-terminal sequence of 43 amino acids was found to be different from any subunit of FcεRI but nearly identical to that of the human melanoma-associated antigen ME491. Therefore, mAb AD1 binds to a surface glycoprotein on RBL-2H3 cells stERICALLY close to the FcεRI but distinct from the recognized subunits of the receptor.

Basophils and mast cells release potent inflammatory mediators following the cross-linking of their high affinity IgE receptors (FcεRI) (1-3). This activation is accompanied by a number of biochemical events including Ca2+ influx, hydrolysis of phosphoinositides, phosphorylation of proteins, and activation of phospholipase A2 (1-3). Recently, the FcεRI components have been purified, and the cDNA for each of the components has been isolated (4-6). Although transfection of COS-7 cells with these components resulted in the expression of receptors on the cell surface, this was not accompanied by any of the signals for cell activation, e.g., intracellular calcium increase or phosphoinositol breakdown (6, 7). Therefore, it is likely that other cell-specific molecules are essential for transmitting the signal to initiate the activation cascade.

Rat basophilic leukemia cells (RBL-2H3) are a useful model for studies of FcεRI and the mechanism of histamine release (8, 9) and for defining other basophil or mast cell surface molecules. A number of monoclonal antibodies (mAb) have been isolated that inhibit IgE binding to RBL-2H3 cells (10-12). The antibodies were of two types; one group bound to the receptor on the cell surface, competed with IgE for binding to the receptor, immunoprecipitated the FcεRI proteins, and the intact mAb released histamine directly from RBL-2H3 cells, while its Fab fragment inhibited histamine release (13). The second type was mAb AA4 that bound to a unique ganglioside on the mast cell/basophil surface that is closely associated with the FcεRI (14). This mAb inhibited 125I-IgE binding to the cells although IgE and other antireceptor mAb did not inhibit the binding of mAb AA4 to the cells. Exposure of cells to this mAb resulted in inhibition of IgE-mediated histamine release.

The present study describes another monoclonal antibody, mAb AD1, that recognized a cell surface protein on RBL-2H3 cells different from the previously described FcεRI components. At high concentrations, this mAb moderately inhibited IgE-mediated histamine release. The protein recognized by this mAb is highly glycosylated with predominantly N-linked oligosaccharides. The N-terminal amino acid sequence of the purified protein was found to be very similar to a human melanoma-associated antigen. This cell surface glycoprotein may play an important role in the biology of mast cells.

EXPERIMENTAL PROCEDURES

Materials—Peptide N-glycosidase (N-Glycanase), endo-α-N-acetylgalactosaminidase (O-Glycanase), and neuraminidase were from Genzyme, Boston, MA. Tunicamycin was from Sigma. [6-3H]Glucosamine (27 Ci/mmol) and [2-3H]mannose (30 Ci/mmol) were from Du Pont-New England Nuclear. All other materials were obtained as described previously (10).

Buffers—Pipes-buffered saline contained 119 mM NaCl, 5 mM KCl, 25 mM Pipes, 5.6 mM glucose, 0.1% bovine serum albumin, 1 mM Ca2+, and 0.4 mM Mg2+ adjusted to pH 7.4. Borate-buffered saline contained 0.2 M borate, 0.15 M NaCl at pH 8.0.

Cells—The RBL-2H3 cells were maintained in culture as described previously (8). The variant RBL-2H3 cell lines and their characteristics have been reported previously (15, 16). Mast cells were obtained by peritoneal lavage of twenty 200-250-g Sprague-Dawley rats and purified (>$95%) by centrifugation on density gradients of Percoll (17). The human cell lines SK-MEL28, SK-MEL31, and HeLa were obtained from the American Type Culture Collection (Rockville, MD).
**Histamine Release Studies**—Histamine release from RBL-2H3 cells in monolayer culture was performed as described previously (18). Briefly, cells were cultured at 10⁶ cells/16-mm diameter well at 37 °C for 18 h. For direct histamine release, the monolayer was washed with Pipers-buffered saline and then incubated with different concentrations of mAb AD1 for 45 min at 37 °C in Pipes-buffered saline. For indirect release, the monolayer was incubated with different dilutions of mAb AD1 at 1 °C in Eagle’s minimum essential medium with Earle’s salts with 2% fetal bovine serum for 2 h, washed with Pipers-buffered saline, and then incubated with 5.0 μg/ml rabbit anti-mouse Ig for 45 min at 37 °C. For the inhibition of IgE-mediated histamine release, cells were sensitized with anti-trinitrophenyl-specific IgG for 2 h, followed by the addition of the indicated concentration of mAb AD1. After the indicated times, the cells were washed and then incubated with antigen (DNase-HSA at 0.01 μg/ml) for 45 min at 37 °C. Control release was from cells that were incubated with IgE antibody and antigen alone. Histamine determinations and calculations were described as previously (19). Histamine release with rat peritoneal mast cells was done as described previously (18).

**Production of the Monoclonal Antibody mAb AD1—BALB/c mice were immunized intramuscularly with 10⁶ RBL-2H3 cells emulsified in Freund’s complete adjuvant. The injection was repeated every 2–3 wks for 6–8 wk. For adoptive transfer into irradiated mice, the spleen cells were flushed with saline. Hybridoma cell lines were selected that secreted antibodies that inhibited IgE-mediated histamine release. Positive cultures were expanded, cloned, and then finally injected into pristane-primed mice to form ascites. The mAb AD1 was purified from ascites by precipitation with 40% ammonium sulfate followed by ion exchange on DE52.

**Production and Purification of Other Mouse mAb**—The mouse monoclonal anti-trinitrophenyl-specific IgE was purified as described previously (21). The mAb BC4, CAS, CDS, AA4, AC4, and BA3 all inhibit IgE binding to RBL-2H3 cells and have been described previously (10, 13). The mAb ER14 and CAS reacts with the component of the FcRI in immunoblots. A polyclonal rabbit antiserum against the α subunit of the FcRI was prepared by immunizing with this component purified by HPLC as described previously (5). Another rabbit anti-α antibody was prepared by injection of the bovine serum albumin-conjugated synthetic peptide STHKQFESILKIQT GKGKKKG that corresponds to the cytoplasmic portion of the α-chain. The antibody was affinity-purified using the same peptide conjugated to CH-Sepharose 4B beads.

**RBL-2H3 Cell Binding Studies**—The various antibodies were ¹²⁵I-labeled with IODO-Beads (Pierce Chemical Co.) and characterized as described previously (10). The mAb AD1 was also ¹²⁵I-labeled by the Bolton-Hunter and iodosulfanilic acid methods (Du Pont-New England Nuclear). Rabbit Ig oligomers were prepared and ¹²⁵I-labeled as described previously (22). Direct binding and inhibition of binding studies were done as described previously (10). For indirect binding, 10⁶ cells were cultured with 10 ng/ml of the indicated antibody in an ice bath. After washing twice with Eagle’s medium essential medium with Earle’s salts containing 2% fetal bovine serum, cells were incubated with ¹²⁵I-rabbit anti-mouse Ig for 90 min at 0 °C. Cells were centrifuged through oil, and the radioactivity in the cell pellet was determined (10). Control binding was done with the same amounts of normal mouse IgG.

**Digestion of Antibodies**—The mAb were digested with pepsin (Boehringer Mannheim GmbH, Germany) or with papain (Worthington) to prepare Fab (‘b) or Fab fragments as described previously (23, 24). The digested materials were purified by HPLC on a Spherogel TSK DBAE 124 column (6.0 mm, inner diameter, 15 cm) followed by gel filtration on a TSK 3000 SWG column (21.5 mm, inner diameter, 60 cm). The purity of the digested products was determined by SDS-PAGE followed by silver staining.

**Immunoprecipitation with mAb AD1**—Immunoprecipitations were done as previously described (10). Briefly, RBL-2H3 or rat peritoneal mast cells were cell surface-labeled with ¹²⁴I by the lactoperoxidase method. The labeled cells were washed twice in ice-cold phosphate-buffered saline containing protease inhibitors and then solubilized in 10 mM Tris, 100 mM NaCl, 1% Nonidet P-40, and 0.2 M sodium phosphate, pH 8.6. After extensive washing with borate-buffered saline containing 2 mM CHAPS and protease inhibitors, the immunoprecipitated proteins were eluted by boiling in SDS-PAGE sample buffer and analyzed by SDS-PAGE, followed by autoradiography.

**Production of the Solubilized Membranes**—The solubilized membranes, purified FcRI proteins, or purified antigen recognized by mAb AD1 were separated by SDS-PAGE using 4–20% gradient gels and transferred to nitrocellulose paper (10). The mAb (10 ng) was incubated with the nitrocellulose, the membranes were washed, and ¹²⁵I-rabbit Fab(‘b) anti-mouse Ig or goat anti-rabbit Ig was added. Following extensive washing, the nitrocellulose membranes were dried and autoradiographed.

**Deglycosylation**—The deglycosylation of the ¹²⁵I-labeled proteins immunoprecipitated by mAb AD1 was done according to the protocol recommended by the manufacturer. RBL-2H3 cells were surface-labeled with ¹²⁴I, solubilized, and reacted with mAb AD1-conjugated Sepharose 4B. Following extensive washing, bound proteins were eluted by boiling the affinity beads in SDS-PAGE sample buffer. The beads were removed by centrifugation. One aliquot of the eluted proteins was incubated for 16 h at 37 °C, with 10 units/ml peptide N-glycosidase F, in 0.17% SDS, 10 mM 1,10-phenanthroline hydrate, 1.6% Nonidet P-40, and 0.2 M sodium phosphate, pH 8.6. Another portion of the eluted proteins was incubated with 1 unit/ml neuraminidase for 2 h at 37 °C, and some of this sample was then incubated for 16 h at 37 °C with 3.0 mill units of endo-β-N-acetylgalactosaminidase in 20 mM Tris maleate, 10 mM D-galactono-β-lactone, 1 mM calcium acetate, 0.15% SDS, and 1% Nonidet P-40. All proteins were then analyzed by SDS-PAGE followed by autoradiography.

**Histamine Release Studies**—Histamine release from RBL-2H3 cells were also cultured for 3 days with 2 μg/ml of living skin. Cell viability of the attached cells was greater than 95% as determined by trypan blue dye exclusion. One aliquot of the cells was surface-labeled with ¹²⁵I and used for immunoprecipitation studies; while another aliquot was used for the preparation of membranes for immunoblotting.

**Immunoprecipitation of the Protein Recognized by mAb AD1—**RBL-2H3 cells were injected subcutaneously into newborn Wistar rats, and the resulting tumors were collected 14 days later. Single cell suspensions were prepared by freezing the tumors through a fine mesh screen in borate-buffered saline containing protease inhibitors and 4 mM EDTA. For the immunoprecipitation experiments, and the bound proteins were eluted with 0.1 M phosphate buffer (pH 6.8) containing 0.5% SDS. The material was then separated by HPLC gel filtration chromatography on columns of SpheroCel TSK 2000SW, 3000SW, and 4000SW (7.5 mm, inner diameter, × 30 cm), and one guard column (SpheroCel SW-pre) connected in series. The flow rate was 0.3 ml/min using a 0.1 M phosphate buffer (pH 6.8) containing 0.2% SDS. Optical density was monitored at 215 and 280 nm, and appropriate samples were analyzed by SDS-PAGE followed by silver staining.

**N-terminal Amino Acid Sequencing**—N-terminal sequence analysis was done with a model 477A Applied Biosystems (Foster City, CA) automated sequenator (25). Stepwise liberated phenylthiobiotin-amino acids were identified using an “on-line” model 120A HPLC with a phenylthiobiotin C18 (2.1 × 220-mm, 5-μm particle) column. The standard method was optimized as described previously (26). Since the sample contained SDS (0.2% in the 50-μl volume loaded on the disc), it was pretreated “in situ” with a small volume of undiluted trifluoroacetic acid using just enough to wet the disc, followed by extensive washing with ethyl acetate and butylic chloride. Computer analysis of the sequence was done with software from the Genetics Computer Group, University of Wisconsin (Version 6.0) (27).

**RESULTS**

**Isolation of mAb AD1—**Spleen cells from mice immunized with RBL-2H3 cells were used for the production of hybridomas. The resulting hybridoma supernatants were tested for their capacity to inhibit IgE-mediated histamine release. It was expected that this assay would detect antireceptor antibodies and other mAb that would inhibit release by interfering with the cell signaling process. For extensive optimization of the antigen, a new mAb was isolated (data not shown). These antibodies had characteristics that were similar to the previously described antireceptor antibodies, i.e., they inhibited ¹²⁵I-IgE binding, released histamine directly, and inhibited the binding of the previously described antireceptor antibody mAb BC4. A number of other mAb were recognized that inhibited histamine release. One of
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these monoclonal antibodies, mAb AD1, is described in detail in this paper. By immunodiffusion, mAb AD1 was found to be of the IgG1 isotype, having a κ chain.

Histamine Release Studies with mAb AD1:—There was no direct or indirect histamine release by mAb AD1 at concentrations up to 100 μg/ml. However, mAb AD1 did inhibit the IgE-mediated histamine release from RBL-2H3 cells in a dose-dependent manner (Fig. 1). The maximum inhibition of 49 ± 10% (x ± S.E., n = 11) was observed with 100 μg/ml mAb AD1.

Binding Studies—The capacity of mAb AD1 to inhibit binding of IgE or other known anti-FcRI antibodies could assist in identifying the site to which it binds on the cell surface. The mAb AD1 at concentrations up to 100 μg/ml did not inhibit the binding of 125I-IgE to RBL-2H3 cells. Furthermore, in RBL-2H3 monolayers cultured with 125I-IgE, the addition of mAb AD1 (16 μg/ml) did not decrease the number of FcεRI nor did it increase the amount of IgE that had been internalized (data not shown). Furthermore, mAb AD1 did not inhibit the binding to RBL-2H3 of the other known antireceptor antibodies mAb BC4, BA3, or AC4 (10). The mAb AD1 also did not bind to the Fcγ receptors known to be present on RBL-2H3 cells (22). The (ab')2 of mAb AD1 at 30 μg/ml did not inhibit 125I-IgG oligomer binding to cells, whereas intact mAb AD1 inhibited this binding (50% inhibition at 1.1 μg/ml). Thus, mAb AD1 did not inhibit the binding of IgE to its receptor, and it did not appear to be directed toward the FcγRI.

The capacity of the mAb AD1 to bind to RBL-2H3 cells was demonstrated by both direct and indirect binding studies. When mAb AD1 at 1 and 10 μg/ml was incubated with 10^6 cells followed by 125I-anti-mouse Ig, there was binding comparable to other immunoglobulin class matched antireceptor antibodies. The binding was less with 1 than with 10 μg/ml, suggesting that mAb AD1 has a low affinity. For direct binding studies, the mAb AD1 was radiolabeled by three different methods, and all gave similar binding curves. At the plateau portion of the curves, there were 1.1 × 10^5 molecules bound per cell (1.1 × 10^5 ± 0.15 × 10^5, n = 7) with a Kd of 4.6 × 10^-8 M/liter (Fig. 2). In parallel experiments, the number of IgE receptors determined using 125I-IgE was approximately 3-fold higher. The extent of labeled mAb AD1 and IgE binding was also determined in 10 variant RBL-2H3 cell lines that have a variable number of FcεRI (15, 16). The number of mAb AD1 molecules bound did not correlate with the number of FcεRI.

Radiolabeled mAb AD1 was also used in binding cross-inhibition studies. IgE did not inhibit 125I-mAb AD1 from binding to the RBL-2H3 cells (Fig. 3). However, the previ-
uously described anti-Fc\textsubscript{RI} antibodies mAb BA3, CD3, and CA5 inhibited mAb AD1 binding. The inhibition curves for mAb BA3, CD3, and CA5 were interesting; they reached plateaus at about 40% inhibition of \textsuperscript{125I}-mAb AD1 binding (Fig. 3A). The addition of more of the mAb at concentrations up to 30 \textmu g/ml did not increase this inhibition. The Fab fragments of these mAb did not inhibit mAb AD1 binding (Fig. 3B), suggesting that the inhibitory effects of the intact mAb could be due to steric hindrance. The mAb AA4, an mAb that binds to a ganglioside in the membrane close to the Fc\textsubscript{RI}, completely inhibited the \textsuperscript{125I}-mAb AD1 binding to RBL-2H3 cells (with 50% inhibition at 0.3 \textmu g/ml). However, mAb AD1 did not inhibit the binding of \textsuperscript{125I}-mAb AA4. Binding inhibition data with mAb AD1 labeled by the three different iodination methods gave similar results. These binding inhibition data were obtained with \textsuperscript{125I}-mAb AD1 at approximately 85% of the saturating concentration; when they were repeated with the mAb AD1 at 50% saturation, there was no significant inhibition by any of the different mAb with the exception of mAb AA4. These data suggest that mAb AD1 binds to surface molecules, only some of which are close to the Fc\textsubscript{RI}.

Immunoprecipitation and Immunoblotting with mAb AD1—The mAb AD1 immunoprecipitated a broad 50–60-kDa band, under both nonreducing and reducing conditions from \textsuperscript{125I}-surface-labeled RBL-2H3 cells (Fig. 4A). Much fainter bands were seen in some experiments at 100–110 kDa. Similar patterns were seen using indirect immunoprecipitation with rabbit anti-mouse Ig coupled to beads or by using a different solubilization procedure with 0.5% Nonidet P-40. Immunoprecipitation of \textsuperscript{125I}-surface-labeled peritoneal rat mast cells showed the same pattern, indicating that this surface protein is found not only on RBL-2H3 cells but also on rat mast cells (Fig. 4B). Since the anti-Fc\textsubscript{RI} mAb BC4 or IgE also immunoprecipitates broad 50–60-kDa bands and because the various anti-Fc\textsubscript{RI} mAb inhibited mAb AD1 binding, it was possible that the mAb AD1 was binding to the Fc\textsubscript{RI}. Therefore, sequential cross-immunoprecipitations were performed (Fig. 5). Surface-labeled cell extracts were preabsorbed twice with mAb BC4 or AD1 or normal mouse IgG beads. The supernatants after the second absorption were each again immunoprecipitated with mAb BC4 or AD1 or normal mouse IgG beads. Preclearing with mAb BC4 beads depleted the Fc\textsubscript{RI} proteins from the extract; however, the band seen with mAb AD1 did not change (Fig. 5, lane 8 compared with lane 14). In the reciprocal experiments, clearning with mAb AD1 did not remove the band precipitated by mAb BC4 (Fig. 5, lane 10 compared with lane 13). This experiment demonstrates that mAb AD1 recognizes a molecule different from the \(\alpha\)-chain of Fc\textsubscript{RI}. Sequential cross-immunoprecipitation was also done using IgE, polyclonal anti-\(\alpha\)-antibodies, and mAb AD1. The results demonstrated that the protein identified by mAb AD1 was distinct from the \(\alpha\) subunit of the Fc\textsubscript{RI} (data not shown).

Immunoblotting with several antibodies was used to examine further the molecules recognized by mAb AD1. Whole cell membranes or purified Fc\textsubscript{RI} proteins were separated by SDS-PAGE and transferred to nitrocellulose. The mAb AD1 bound to a 50–60-kDa band on cell membranes but not to Fc\textsubscript{RI} proteins (Fig. 6, lane A3 versus B3). The mAb ER14 CA4 is an anti-\(\alpha\)-antibody that immunoprecipitates the \(\alpha\)-chain and also binds to it in immunoblots. This mAb bound to similar bands on both cell membranes and on the Fc\textsubscript{RI} proteins (Fig. 6, lanes A2 and B2). Therefore, the molecule recognized by mAb AD1 is not present in the purified Fc\textsubscript{RI} proteins but is found in the membrane preparations and is clearly distinct from the \(\alpha\) component of the Fc\textsubscript{RI} on RBL-2H3 cells.

The Protein Recognized by mAb AD1 Is Highly Glycosylated—The broad bands observed in the immunoprecipitation experiments suggested that the antigen recognized by mAb AD1 is a glycosylated protein. Biosynthetic labeling studies with [\(6\textsuperscript{3}H\)]glucosamine and [\(2\textsuperscript{3}H\)]mannose followed by immunoprecipitation with either mAb AD1 or with the anti-receptor antibody mAb BC4 resulted in the precipitation of similar 50–60-kDa bands. These findings confirmed that the surface protein precipitated by mAb AD1 is glycosylated. Enzymatic deglycosylation experiments were undertaken to define further the extent of the carbohydrates on the antigen precipitated by mAb AD1. The protein immunoprecipitated by mAb AD1 was isolated from a 10 mM CHAPS lysate of \textsuperscript{125I}-surface-labeled RBL-2H3 cells and treated with peptide N-glycosidase F to cleave the N-linked oligosaccharides. As shown in Fig. 7A, this treatment transformed the broad 50–60-kDa band into a sharp 27-kDa band. In contrast, the Fc\textsubscript{RI} \(\alpha\)-chain was relatively insensitive to treatment with peptide N-glycosidase F and displayed a different pattern. To define the role of O-glycosylation, the AD1 antigen was first incubated with neuraminidase to cleave the accessible sialic acid residues and then subsequently treated with endo-\(\alpha\)-N-acetylgalactosaminidase to hydrolyze N-acetylgalactosamine linked to serine residues. The neuraminidase treatment had a very minor effect on the 50–60-kDa protein, and there was essentially no change after treatment with endo-\(\alpha\)-N-acetylgalactosaminidase (Fig. 7A). In contrast, the Fc\textsubscript{RI} \(\alpha\)-chain was not affected by treatment with either neuraminidase nor endo-\(\alpha\)-N-acetylgalactosaminidase (data not shown). These data therefore indicate that N-linked carbohydrates are a major part of this glycoprotein.

Tunicamycin, an inhibitor of the addition of N-acetylgalactosamine to proteins, was used to study the synthesis of this antigen. RBL-2H3 cells were cultured with 2 \mu g/ml tunicamycin for 3 days and then used either for immunoprecipita-

![Fig. 4. Immunoprecipitation of proteins by mAb AD1 from \textsuperscript{125I}-surface-labeled RBL-2H3 cells (A) or from I-surface-labeled rat peritoneal mast cells (B). A, the cells were surface-labeled with \textsuperscript{125I} as described under "Experimental Procedures," and the solubilized cell extracts were incubated with mAb AD1-coupled Sepharose 4B beads. After washing, the immunoprecipitated proteins were eluted and separated by SDS-PAGE under nonreducing and reducing conditions on 4–20% gradient gels and then autoradiographed. Lanes: 1 and 3, mAb AD1; 2 and 4, IgG; 1 and 2, nonreduced; 3 and 4, reducing conditions. B, rat peritoneal mast cells were purified and processed as above. Lane 1, mAb AD1 under nonreducing conditions; lane 2, IgG.](image-url)
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Fig. 5. Sequential cross-immunoprecipitation with mAb BC4 and mAb AD1. RBL-2H3 cells were surface-labeled with $^{125}$I and solubilized, and the extracts were incubated twice with mAb BC4, mAb AD1, or IgG-coupled Sepharose 4B (lanes 1–6). Each supernatant was then divided into three aliquots and incubated with either mAb BC4, mAb AD1, or IgG-coupled Sepharose 4B (lanes 7–15). After washing, the immunoprecipitated proteins were eluted, and equal amounts were analyzed by SDS-PAGE on 14% gels under nonreducing conditions followed by autoradiography. The scheme in the right half is the procedure used; the lane numbers correspond to the analysis of the bound proteins at each step.

Fig. 6. Immunoblot analysis with mAb AD1. RBL-2H3 cell membranes (A) or purified FcεRI (B) were transferred to nitrocellulose and incubated with different mAb at 10 μg/ml. Lanes: 1, normal mouse IgG; 2, mAb ER14 CA4, an anti-α mAb; 3, mAb AD1.

Fig. 7. The glycoprotein nature of the protein recognized by mAb AD1. A, deglycosylation of the immunoprecipitated material. RBL-2H3 cells were $^{125}$I-surface-labeled, and the immunoprecipitated proteins were digested with the different enzymes as follows: lanes 1 and 3, untreated mAb AD1 immunoprecipitate; lane 2, incubated with peptide N-glycosidase F; lane 4, treated with neuraminidase; lane 5, treated with neuraminidase followed by endo-α-N-acetylgalactosaminidase. B, RBL-2H3 cells were cultured with 2 μg/ml tunicamycin for 3 days and then $^{125}$I-surface-labeled, solubilized, and immunoprecipitated with mAb AD1 (lane 1) or normal mouse IgG (lane 2). C, immunoblot of membrane proteins prepared from cells cultured with tunicamycin. Lane 1, mAb AD1; lane 2, normal mouse IgG.

Fig. 8. Analysis of the purified glycoprotein. The glycoprotein was affinity-purified by mAb AD1, followed by HPLC size exclusion chromatography. A, SDS-PAGE followed by silver staining of the purified material. B, immunoblot analysis of the purified protein. Lane 1, normal mouse IgG; lane 2, anti-FceRI mAb ER14CA4; lane 3, mAb AD1.
immunoprecipitation (Fig. 8B). In contrast, monoclonal and polyclonal anti-FcR1 antibodies did not bind to this purified material. There was no appreciable difference in the migration of the protein when analyzed by SDS-PAGE under reducing and nonreducing conditions. The Fab fragments did not block the binding of mAb AD1 to the purified protein after it had been reduced and transferred to nitrocellulose paper, indicating the importance of the disulfide groups to the configuration of the mAb binding epitope. Two aliquots from the same batch of the purified protein were used for N-terminal amino acid sequencing. The initial yields were 40 and 46 pmol, respectively, with a dramatic decrease in yield after cycle 8. However, useful sequence information was obtained for a total of 43 cycles (Fig. 9). By a search of the GenBank Release 62.0 data base, the sequence of the protein recognized by mAb AD1 was found to be very similar to the melanoma-associated antigen ME491 present on human nonmetastatic melanoma cells (28). The similarity of the N-terminal sequences is striking especially in view of the fact that one is from rat and the other from human tissues. Further evidence of the similarity of the two proteins was obtained by demonstrating the binding of mAb AD1 to the human SK-MEL-28 melanoma and HeLa cell lines that are known to express this ME491 protein (28). The mAb AD1 specifically immunoprecipitated a broad 35-85-kDa protein from 125I-labeled surface-labeled SK-MEL-28 and SK-MEL-31 human melanoma cells. Therefore, the protein recognized by mAb AD1 is probably very similar to the human melanoma antigen ME491.

**DISCUSSION**

The present study describes the use of mAb AD1 to characterize a novel surface protein on RBL-2H3 cells. Both by immunoprecipitation and immunoblotting, mAb AD1 bound to a 50-60-kDa protein band that is distinct from the α component of the FcR1.

The FcR1 on RBL-2H3 cells consists of three non-covalently linked components (7). The IgE binding α subunit is a 45-60-kDa transmembrane protein; most of this protein is on the outside of the cell. Although the protein immunoprecipitated by mAb AD1 shows a migration and apparent molecular weight on SDS-PAGE similar to those of the α subunit, the experimental evidence indicates that they are different proteins. The binding studies, the sequential immunoprecipitation findings, and the N-terminal amino acid sequencing results indicate that mAb AD1 binds to a membrane glycoprotein that is not part of FcR1.

The binding cross-inhibition data suggest that the mAb AD1 binds to a site close to the FcR1. The site is distant enough so that there was no cross-inhibition in binding with IgE. However, the other antireceptor antibodies inhibited up to 40% of mAb AD1 binding by intact molecules, although the Fab fragments did not block the binding of mAb AD1. Because the Fab fragments of these molecules did not inhibit mAb AD1 binding, the inhibitory effect of the intact molecules was probably due to steric hindrance. The partial inhibition of mAb AD1 binding could suggest that only some of the molecules on the cell surface to which mAb AD1 bind are close to the FcR1.

The mAb AD1 does not bind to the FcγR on RBL-2H3 cells. These cells have surface FcγR that bind both IgG and IgE with low affinity (22). Intact mAb AD1 inhibited IgG oligomer binding, but the F(ab')2 was inactive. However, the F(ab')2 still was active in inhibiting histamine release. Therefore, it is unlikely that the mAb AD1 binds to IgG receptors on the cell surface.

A number of antibodies have been described that inhibit histamine release from RBL-2H3 cells (10). One of these antibodies, mAb AA4, recognizes a unique ganglioside present on RBL-2H3 cells (14) and inhibits 51I-IgE binding. The mAb AA4 inhibits both IgE- and ionophore-mediated histamine secretion by a mechanism independent of its inhibition of IgE binding. The characteristic effects of the mAb AD1 are different from those of mAb AA4. The mAb AD1 immunoprecipitated a unique protein from the cell surface and did not inhibit IgE binding. The mAb AA4 also inhibited the binding of mAb AD1. This suggests that the glycoprotein recognized by mAb AD1 and the ganglioside are close on the cell surface.

Several other mAb have been isolated following immunization with partially purified FcγR1 (11). These mAb clearly did not precipitate the same proteins as mAb AD1. Recently, another mAb (G63) was reported to inhibit serotonin secretion from RBL-2H3 cells (29). This mAb immunoprecipitated a disulfide-linked 55-70-kDa glycosylated protein from surface-labeled cells that decreased to 28-40 kDa under reducing conditions. In contrast, the mAb AD1 immunoprecipitated a 50-60-kDa protein under both reducing and nonreducing conditions. Therefore, the protein recognized by mAb AD1 appears to be different from those previously identified with other antibodies.

The N-terminal sequence of the glycoprotein recognized by mAb AD1 was very similar or identical to the ME491 melanoma-associated antigen. This similarity is remarkable in view of the fact that the comparison is between human and rat proteins (Fig. 9). The ME491 antigen migrated as a broad 30-60-kDa band and was found on a number of human adenosarcomas as well as some normal secretory cells (28). The ME491 glycoprotein was thought to be present in large amounts during the early stages of melanoma development but disappeared when the cells became metastatic (28). By immunohistochemical staining of human tissues, the mAb that reacted with ME491 bound to normal secretory cells, e.g. cells in the adrenal medulla, pituitary, salivary, and thyroid glands. Some, but not all, authors have noted the presence of the ME491 antigen on mast cells (30-32). Nucleotide sequence analysis of the cloned complementary DNA indicated that the ME491 antigen consists of 237 amino acids (M, 25,475) with four transmembrane regions. The gene for ME491 mapped to the human chromosome region 12q12-12p14. ME491 contains 14 cysteine residues that are probably important for its structural integrity. Both ME491 and the protein precipitated by mAb AD1 have similar molecular weights and are heavily N-glycosylated. Accordingly, mAb AD1 immunoprecipitated broad 50-60-kDa bands from the human melanoma cell line SK-MEL-28 and from HeLa cells. The ME491 sequence has no homology to known proteins important for signal transduction such as those in the immunoglobulin gene superfamily or G-protein family. The ME491 sequence suggests four putative transmembrane regions which could indicate that it is a ligand-gated ion channel, although there it has no homology to known ion channels (28). The N-glycosylation of this protein might suggest that it plays a role as an adhesion molecule critical for cell differentiation. Several investigators have emphasized the neuroendocrine distribution of this antigen and postulated that it might have a role in the secretory process (30, 31). The fact that the N-terminal sequences of the rat and human proteins are nearly identical suggests

FIG. 9. The N-terminal amino acid sequence of the protein purified by mAb AD1 from RBL-2H3 cells and its comparison with the human melanoma antigen ME491.
strong selective pressure to maintain this sequence. Therefore, it appears likely that this molecule has an important function. Further efforts are needed to define whether this protein has any membrane channel function or plays a role in cell differentiation.

In conclusion, we have characterized a mAb that binds to a novel protein on RBL-2H3 cells that is located close to the FcRI. The relationship of the protein recognized by mAb AD1 to the topography and function of the FcRI remains to be studied.

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