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Immunology

Structural homology between lymphocyte receptors for high endothelium and class III extracellular matrix receptor

(cell adhesion/lymphocyte recirculation/collagen/proteoglycan)

W. MICHAEL GALLATIN*†, ELIZABETH A. WAYNER*, PATRICIA A. HOFFMAN*, TOM ST. JOHN*, EUGENE C. BUTCHER‡, AND WILLIAM G. CARTER*

*Fred Hutchinson Cancer Research Center, Division of Basic Sciences, 1124 Columbia Street, Seattle, WA 98040; and ‡Department of Pathology, Stanford University, Stanford, CA 94305

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ABSTRACT **We have identified extensive structural homology between one type of heterotypic adhesion receptor (HAR) involved in lymphocyte interactions with high endothelium in lymphoid organs and a collagen-binding protein, termed class III extracellular matrix receptor (ECMRIII), expressed on most nucleated cell types. Both receptors have been described as heterogeneous 90-kDa transmembrane glycoproteins, referred to here as gp90. Monoclonal anti-HAR antibodies, Hermes-1 and Hutch-1, and monoclonal anti-ECMRIII antibodies, P1G12 and P3H9, were utilized to compare the two receptors. (*i*) All these monoclonal antibodies immunoprecipitated major gp90 components as well as uncharacterized additional higher molecular mass antigens of 120–200 kDa in human and macaque fibroblasts and peripheral blood mononuclear cells. (*ii*) Competitive binding analyses with the antibodies identified distinct epitopes present on gp90. (*iii*) Enzymatic and chemical digestions generated identical peptide fragments from all the antigens in human and macaque fibroblasts and peripheral blood mononuclear cells. (*iv*) Sequential immunoprecipitation with P1G12 followed by the other monoclonal antibodies indicated that all gp90 species reactive with Hermes-1 and Hutch-1 also expressed the P1G12 defined epitope. In reciprocal experiments, Hermes-1 and Hutch-1 immunoprecipitation did not completely remove all P1G12-reactive gp90 from cellular extracts. One inference from these data would be that gp90 is serologically heterogeneous, encompassing HARs as a major subset of this broadly expressed class of molecules.**

Previously a heterogeneous 90-kDa membrane glycoprotein that binds to type I and VI collagen in affinity chromatography experiments has been identified in detergent extracts of HT1080 human fibrosarcoma cells; it has been termed the class III extracellular matrix receptor (ECMRIII) (1, 2). Distribution studies with the anti-ECMRIII monoclonal antibodies (mAbs) P1G12 and P3H9 (2) revealed that ECMRIII is expressed in many nucleated cells, including lymphoid, myeloid, epithelial, glial, and fibroblast populations, but was not detectable in either erythrocytes or platelets. From structural analyses, a model for ECMRIII was presented that postulated a 65-kDa core protein, which was heavily modified posttranslationally containing serine-phosphate residue(s) on the cytoplasmic domain, a hydrophobic transmembrane domain, and a highly glycosylated extracellular domain containing both asparagine and unidentified carbohydrate linkages. ECMRIII was also found to codistribute with the cytoskeleton. None of the antibodies prepared against ECMRIII inhibited cell adhesion to collagen or other extracellular matrix (ECM) components, suggesting either that ECMRIII was not directly involved in cell adhesion to the ECM or that the mAb-defined epitopes examined were not directly involved in this function.

In separate studies, 90-kDa glycoproteins (referred to as gp90s) had been implicated in adhesion of lymphocytes to high endothelial venules (HEV). Three different mAbs defined these heterotypic adhesion receptors (HARs) in mouse (MEL-14; ref. 3), human (Hermes-1; ref. 4) and macaque (Hutch-1; see below). Although implicated in similar functions, the MEL-14 antigen and the gp90s defined in primates are not structural homologues (5, 6). During exit from the bloodstream, lymphocytes have been proposed to interact with ligands on the surface of high endothelium referred to as "vascular addressins" (7). It has also been proposed that one murine 90-kDa HAR, bound by the MEL-14 antibody, is a lectin with binding specificity for phosphorylated mannose residues (8). Recent results (9) have indicated that molecules closely related or identical to the Hermes-1 antigen originally defined in human lymphoid cells are also expressed by other human cells of nonhemopoietic origin.

Because of the apparent structural similarities in molecular mass and isoelectric point between ECMRIII and the glycoproteins recognized by Hermes-1 and Hutch-1, we undertook a direct comparison of the two. Here, we have examined the biochemical characteristics of ECMRIII and the primate HARs and have concluded that they share partial or complete structural homology. (*i*) Utilizing competitive antibody binding, we have defined multiple epitopes carried by both receptors. (*ii*) Peptide mapping also indicates structural homology between these glycoproteins on both human and macaque cells. (*iii*) Sequential immunoprecipitation experiments indicate that mAbs P1G12 and P3H9 to ECMRIII define a heterogenous and widely expressed family of molecules that encompass the glycoproteins recognized by the Hermes-1 and Hutch-1 mAbs.

MATERIALS AND METHODS

Materials. Phenylmethylsulfonyl fluoride, *N*-ethylmaleimide, diisopropyl fluorophosphate, 2-mercaptoethanol, bovine serum albumin, Triton X-100, EDTA, and protein A-agarose were purchased from Sigma. Lactoperoxidase and glucose oxidase were from Calbiochem. Rabbit anti-mouse IgG (heavy and light chain) antiserum was obtained from Cappel Laboratories (Cooper Biomedical). ^{125}I was from Amersham.

Cells and Cell Culture. The human fibrosarcoma cell line HT1080 was obtained from the American Type Culture Collection. Peripheral blood mononuclear cells (PBMC) from

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Abbreviations: ECMRIII, class III extracellular matrix receptor; HAR, heterotypic adhesion receptor; PBMC, peripheral blood mononuclear cells; mAb, monoclonal antibody; ECM, extracellular matrix; FITC, fluorescein isothiocyanate.

†To whom reprint requests should be addressed.

normal human and macaque (*Macaca nemestrina*) donors were purified on Ficoll/Hypaque (density = 1.077 g/ml) according to a standard protocol (10).

Antibodies. Monoclonal anti-ECMRIII antibodies P1G12 and P3H9 were produced by the methods of Oi and Herzenberg (11) and Taggart and Samloff (12) as described in detail (2). The production of Hermes-1, a rat IgG2a mAb, has been described (4). Hutch-1 is a mouse IgG2a mAb derived from fusion of NS-1 myeloma cells to spleen cells from a BALB/c mouse immunized with whole macaque PBMC. The initial selection of the hybrid-secreting Hutch-1 was detected with a mouse immunoglobulin-specific fluorescein isothiocyanate (FITC)-conjugated second stage reagent (Southern Biotechnology Associates, Birmingham, AL). Its distribution in PBMC was coincident with that of Hermes-1 with the exception of rare Hermes-1^+/Hutch-1^- cells. Within the limits of resolution, the Hutch-1 and Hermes-1 antigens were found to have identical distributions on macaque lymphoid tissue in subsequent immunohistologic tests. Hutch-1 was found to recognize a gp90 that was completely and specifically depleted from surface-radioiodinated macaque PBMC lysates by sequential immunoprecipitation with Hermes-1. Unlike Hermes-1, Hutch-1 does not react detectably in either flow cytometric tests or in immunoprecipitation analyses with human cells and is apparently macaque specific.

Immunoprecipitation and Sequential Immunoprecipitation. Cells were surface labeled with radioactive iodine utilizing the lactoperoxidase/glucose oxidase method (13), followed by extraction with 1% (vol/vol) Triton X-100 in 25 mM Tris·HCl at pH 7.5 containing 1 mM diisopropyl fluorophosphate or 1 mM phenylmethylsulfonyl fluoride plus 2 mM *N*-ethylmaleimide as protease inhibitors. Immunoprecipitation and sequential immunoprecipitation were performed as previously described (1). For macaque lysates examined by sequential immunoprecipitation, an aliquot of each sample was also precycled with an irrelevant mAb, C12, against glycoproteins unrelated to gp90. This group served as a negative control for nonspecific antigen removal due to precycling. Similarly, cell lysates immunoprecipitated for tryptic and chymotryptic digestion were first subjected to three cycles of preclearance with C12. The relative abundance of glycoproteins isolated subsequentially with Hutch-1, Hermes-1, and P1G12 was not affected selectively by this procedure (see Fig. 3 *a* and *b*).

Peptide Analysis by Digestion with Trypsin, Chymotrypsin, and Cyanogen Bromide. Peptide analysis was as previously described (2).

PAGE. SDS/PAGE slab gels were prepared following the basic stacking gel system of Laemmli (14) and as described (1). Prestained protein standards for relative molecular mass estimation were obtained from Bethesda Research Laboratories and were as follows: lysozyme (14.3 kDa), β-lactoglobulin (18.4 kDa), chymotrypsin A (25.7 kDa), ovalbumin (43.0 kDa), bovine serum albumin (68 kDa), phosphorylase B (97.4 kDa), and myosin heavy chain (200 kDa).

Flow Cytometric Analyses. Macaque PBMC obtained by means of density gradient centrifugation were incubated with anti-ECMRIII or anti-HAR mAbs and subjected to flow cytometric analyses on either a modified Becton Dickinson FACS II or a Coulter EPICS 750-II instrument. Photomultiplier voltages and gains were standardized using CaliBRITE beads (Becton Dickinson). For the data reported here, gates were set on forward angle and 90° light scatter to exclude dead cells, erythrocytes, and most monocytes and granulocytes from the analysis. Uptake of propidium iodide measured by emission >620 nm after 488 nm excitation was used as an additional criterion for dead cell exclusion. Histograms presented in Fig. 2 correspond to a four-decade logarithmic scale. The basic protocol for competitive inhibition experiments was as follows. PBMC were washed, at 4°C, three times in Hanks' balanced salt solution with 5% fetal calf serum and 10 mM sodium azide (HBSS-5). Aliquots of 10^6 cells were then incubated on ice for 30 min with unconjugated anti-ECMRIII or anti-HAR reagents. Biotin conjugates of either Hermes-1, P3H9, Hutch-1, or P1G12 were then added and the samples were incubated for an additional hour and washed three times in HBSS-5. After an additional 20-min incubation with FITC-avidin (Vector) with 10 μg of propidium iodide per ml, samples were washed three times and resuspended at 5×10^6 per ml for analysis. Each labeled and unlabeled reagent was used in at least a 2-fold excess above that required to give saturation.

RESULTS

Shared Epitopes Expressed by ECMRIII and HARs. The antigens recognized by the anti-ECMRIII mAbs P1G12 and P3H9 and the anti-HAR mAbs Hermes-1 and Hutch-1 were compared by immunoprecipitation of antigens from Triton X-100 detergent extracts of ^{125}I surface-labeled human and macaque PBMC and human HT1080 fibrosarcoma cells (Fig. 1). Anti-ECMRIII and Hermes-1 mAbs immunoprecipitate similar 90-kDa membrane proteins in all three cell populations. The Hutch-1 antibody also precipitates a gp90, but only from macaque PBMC. Multiple forms of the precipitated antigens were detected in both the macaque and human cells (Fig. 1). Macaque PBMC contained two other high molecular mass species, of 200 and 180 kDa, respectively. Molecules of this size were not readily detected in the human cells. HT1080 cells contained multiple relatively minor antigens of 120, 150, and 170 kDa. The precise structural basis for cross-reactivity between the gp90s and these other antigens is unknown at this time.

The possibility that the antigens recognized by both the anti-ECMRIII and anti-HAR mAbs were homologous membrane components was investigated in subsequent studies. The ability of these antibodies to competitively inhibit bind-

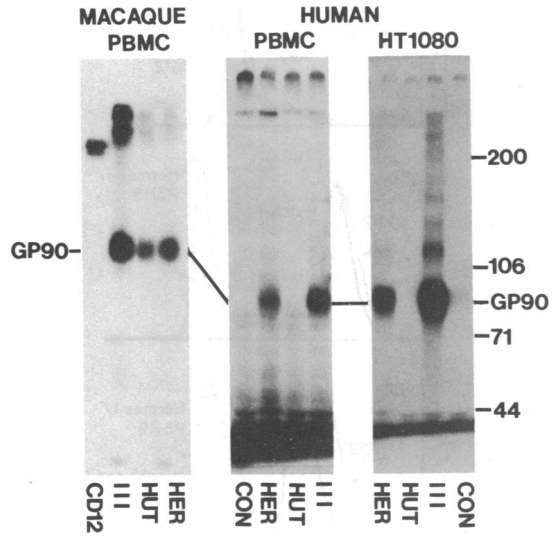

FIG. 1. Comigration of Hermes-1, Hutch-1, and ECMRIII antigens immunoprecipitated from macaque and human cells. Macaque PBMC, human PBMC, and HT1080 human fibrosarcoma cells were extracted with Triton X-100 after cell surface labeling with ^{125}I. The extracts were immunoprecipitated with the mAbs Hermes-1 (HER), Hutch-1 (HUT), and P1G12 (III) or with control SP2 cell culture supernatant (CON) or irrelevant CD12 mAbs (CD12) as indicated. The immunoprecipitates were fractionated by SDS/PAGE (8% gels for human antigens and 9% gels for macaque antigens) and detected by autoradiography. Migration of prestained molecular mass markers are indicated at the right. Migration of the major 90-kDa antigen precipitated by the specific antibodies is labeled gp90.

ing of each other to macaque PBMC was assessed by flow cytometric analysis. Following incubation with each unlabeled reagent, biotin conjugates of either Hermes-1 (Fig. 2*A*), P3H9 (Fig. 2*B*), Hutch-1 (Fig. 2*C*), or P1G12 (Fig. 2*D*) were added to each sample. The effect of each pretreatment on subsequent binding of biotinylated antibodies was quantitated by flow cytometric analysis with avidin-FITC as a final indicator of bound biotinylated antibody. The anti-HAR and anti-ECMRIII reagents react with serologically related molecules, in some cases binding to overlapping epitopes. For example, Hermes-1 blocked not only subsequent binding of additional Hermes-1 but also that of P3H9 (Fig. 2 *A* and *B*). Conversely, Hermes-1 did not block Hutch-1 or P1G12 staining (Fig. 2 *C* and *D*). The binding of Hutch-1 was inhibited by preincubation with P1G12 (Fig. 2*C*). Reciprocally, P1G12 was blocked to a large extent but not completely by prebinding Hutch-1 (Fig. 2*D*). Neither of these two mAbs affected binding of Hermes-1 or P3H9 (Fig. 2 *A* and *B*). It seems likely that P1G12 and Hutch-1 define qualitatively distinct but spatially overlapping epitopes since the former reagent reacts with both human and macaque cells, whereas the latter is macaque specific (Figs. 1 and 3). Thus, at least two and perhaps three independent epitopes are defined by the binding of P3H9/Hermes-1, Hutch-1, and P1G12 mAbs.

Mapping of ECMRIII and HARs. Further indication of the homology between the HARs and ECMRIII was obtained by

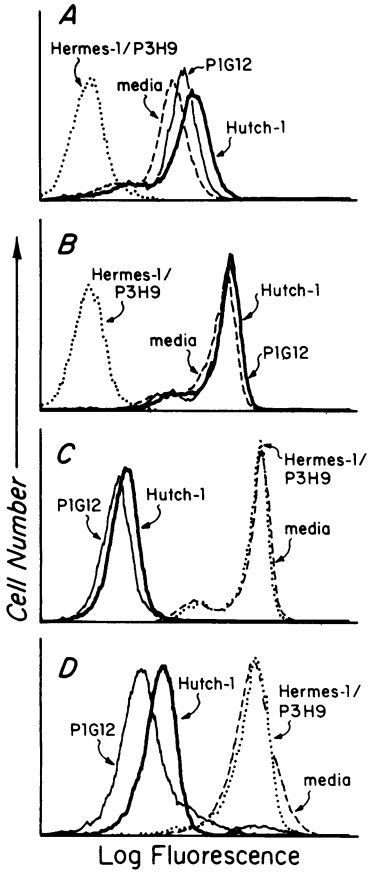

FIG. 2. Cross-competition of anti-HAR and anti-ECMRIII antibodies for epitopes expressed by macaque PBMC. PBMC were incubated with a saturating amount of unconjugated Hermes-1, P3H9, Hutch-1, or P1G12 or with no antibody (media). After this a saturating amount of biotinylated Hermes-1 (*A*), P3H9 (*B*), Hutch-1 (*C*), or P1G12 (*D*) was added. The binding of biotin-mAbs was then quantitated by flow cytometry following the addition of FITC-avidin. Hermes-1 and P3H9 cross-compete for cellular binding sites. Hutch-1 and P1G12 detect epitopes that overlap spatially and are distinct from those bound by Hermes-1 and P3H9.

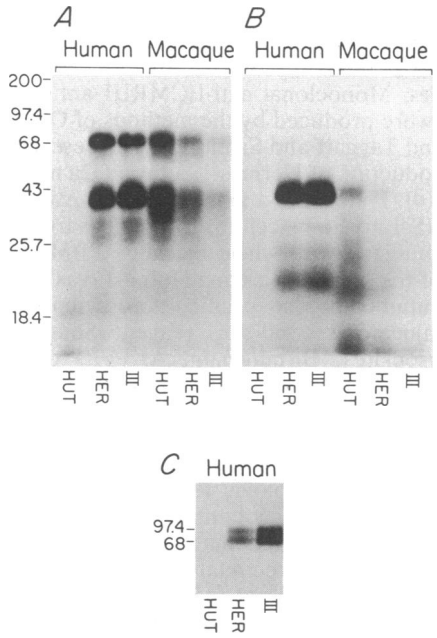

FIG. 3. The Hutch-1, Hermes-1, and ECMRIII gp90 antigens from human and macaque monkey PBMC are homologous as determined by digestion with trypsin (*A*), chymotrypsin (*B*), and cyanogen bromide (*C*). gp90 antigens were purified from extracts of ^{125}I surface-labeled human and macaque PBMC by immunoprecipitation. The purified antigens were partially digested with trypsin, chymotrypsin, or cyanogen bromide followed by fractionation on SDS/15% PAGE gels and detection by autoradiography. Migration of prestained molecular mass markers are indicated at left. Except for minor species-specific differences between human and macaque antigens, the peptide patterns generated for all three antigens were identical.

peptide mapping following trypsin, chymotrypsin, and cyanogen bromide digestion. ECMRIII and HAR were purified by immunoprecipitation with P1G12, Hutch-1, and Hermes-1 from Triton X-100 extracts of ^{125}I/lactoperoxidase-labeled macaque PBMC followed by further purification by preparative gel electrophoresis. As previously reported (2), trypsin cleavage of ^{125}I-labeled ECMRIII generates surface-labeled peptides of ≈27 and 35 kDa that include the extracellular and transmembrane domains, respectively (Fig. 3*A*). The peptide maps obtained from partial tryptic digests (Fig. 3*A*) were qualitatively identical for all three antigens. Limited digestion with chymotrypsin also generated equivalent products in all cases (Fig. 3*B*). Finally, partial cleavage with cyanogen bromide of Hermes-1- and P1G12-precipitated material from human cells yielded a 68-kDa labeled peptide in both cases. By these three independent criteria then, some degree of structural homology is suggested between these antigens.

Sequential Immunoprecipitation of ECMRIII and HAR. Immunoprecipitation of ECMRIII and the HARs routinely detected differences in the relative amounts of labeled antigen recovered from cell extracts (see Fig. 1, for example). To more formally establish that anti-ECMRIII and anti-HAR reagents detect the same or overlapping subsets of glycoproteins, sequential immunoprecipitations were performed. gp90 antigen carrying each epitope was quantitatively removed from labeled cell extracts by multiple cycles of immunoprecipitation with P1G12, Hermes-1, or Hutch-1. Each of these precycled extracts was then immunoprecipitated separately with each mAb to identify any remaining gp90 species. As seen in Fig. 4 *Right*, precycling with P1G12 removed all detectable P1G12 and Hermes-1 antigens from the extracts of HT1080 cells. In contrast, precycling with Hermes-1 failed to remove all P1G12 antigen. Similar results

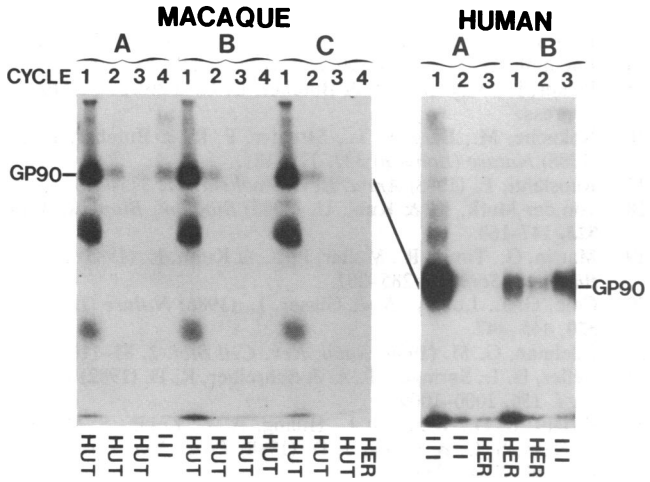

FIG. 4. Sequential immunoprecipitation of gp90 with Hermes-1, Hutch-1, and P1G12 mAbs: possible differential expression of epitopes. Macaque PBMC and human HT1080 cells were surface labeled with ^{125}I and extracted with Triton X-100. Aliquots of the extract were subjected to multiple cycles of immunoprecipitation as follows. (*Left*) mAb Hutch-1 (HUT) was used to precipitate all Hutch-1 antigen from three identical samples (samples A, B, and C) of cell extract with three sequential cycles (cycles 1–3) of immunoprecipitation. For cycle 4, the Hutch-1-free extract was reimmunoprecipitated with P1G12 (III, sample A), Hutch-1 (HUT, sample B), or Hermes-1 (HER, sample C) antibodies to immunoprecipitate any remaining gp90. (*Right*) mAbs P1G12 (III, sample A) and Hermes-1 (HER, sample B) were used to quantitatively remove their respective antigens from two aliquots of the cell extract by two immunoprecipitation cycles each. The precleared extracts were then subjected to a third cycle of immunoprecipitation with the indicated antibodies. Antigens precipitated in the multiple precipitation cycles were analyzed on SDS/PAGE gels (macaque, 10% gel; human, 8% gel) followed by autoradiography. Migration of the gp90 antigen is indicated. Additional undesignated bands precipitated from macaque PBMC correspond to irrelevant heavy and light chains of immunoglobulin immunoprecipitated nonspecifically.

were obtained with sequential immunoprecipitation of gp90 from extracts of macaque monkey PBMC (Fig. 4 *Left*). Again, a detectable amount of P1G12-reactive gp90 remained after exhaustive precycling with the anti-HAR reagents. This finding could mean that HAR glycoproteins are a serologically distinct subpopulation of all the gp90 molecules detected. An alternative explanation for these results would be that the different antibodies bind an identical gp90 antigen with different affinities and/or epitope stabilities and therefore exhibit different efficiencies in immunoprecipitation.

DISCUSSION

Lymphoid HARs and ECMRIII Are Homologous. We have utilized peptide mapping, competitive antibody binding, and sequential immunoprecipitation to show that the 90-kDa lymphoid HARs are closely related to the collagen-binding ECMRIII membrane glycoprotein(s). It will be intriguing if individual gp90 molecules can be demonstrated to carry a dual binding specificity for collagen in the ECM and a cell surface ligand, a vascular addressin, for example. This possibility is supported by the demonstration that isolated Hermes-1 antigen from some nonhematolymphoid cells, including fibroblasts, retain the ability to bind mucosal vascular addressin (15), a potential ligand for lymphoid HARs (7, 16). The ability to bind to multiple ligands is characteristic of ECM components including fibronectin, laminin, and collagen (for a general review, see refs. 17–19). Similarly, neural cell adhesion molecule (20, 21) and Mac-1/CR3 (which can bind complement) (22, 23) have both been shown to interact with multiple ligands. Also, while not directly demonstrated here, the high degree of overlap between anti-ECMRIII-, Hermes-1-, and Hutch-1-reactive species suggests that at least some of the lymphoid HARs may also bind collagen. In a more global sense, the relation of ECMRIII to the lymphoid HARs is interesting since EMCRIII has been shown to be expressed by many nucleated cell types, including lymphoid, myeloid, endothelial, epithelial, neural, and fibroblast cell populations, suggesting that any possible role as a HAR may apply to many different cell populations. The broad tissue distribution of HAR/ECMRIII gp90s implies other as yet undefined physiologic roles for this class of molecules. Furthermore, ECMRIII has been shown to codistribute with components of the cytoskeleton and to contain a major cytoplasmic phosphorylation site (2). These characteristics may be relevant to receptor function in intercellular adhesion beyond that involved specifically with lymphocyte recirculation.

Possible Heterogeneity in Epitope Expression by gp90. Our results indicate that the four antibodies define three different epitopes on gp90 and that these epitopes may be differentially expressed. In human and macaque cells, both P3H9 and P1G12 precipitated antigens that were not recognized by either Hermes-1 or Hutch-1 antibodies. The structural basis for this observed heterogeneity in antibody reactivity has yet to be explained, but it may include one or more of the following: (*i*) heterogeneity in glycosylation including N-linked and other carbohydrate moieties, (*ii*) variation in phosphorylation as previously reported for ECMRIII (2), and (*iii*) variation in the reduced/oxidized status of intramolecular disulfide bonds in gp90 that mediate reactivity with P1G12 and P3H9 antibodies (W.G.C. and E.A.W., unpublished results).

Relation to Work Done by Others. An examination of recent literature indicates that the homology we have observed in HARs and ECMRIII may also extend to additional membrane glycoproteins described by other groups. These possible homologies may be initially obscured by extensive polymorphism in the antigens. Hermes-1-reactive antigens of 180-200 kDa on human lymphocytes have been reported and shown to consist of a 76-kDa core glycoprotein (possibly a common precursor of the 90-kDa and 180-kDa forms) linked to chondroitin sulfate moieties (24). One might reasonably expect that the mouse Pgp-1 antigen (25) and the related p85 protein (26) as well as the CD44 antigen (27), which exhibit similar polymorphism, are related to the gp90s that we have analyzed (HARs and ECMRIII). Recently, in structural comparisons similar to those reported here, this has been confirmed for CD44 and Pgp-1 (9). On the other hand, we could find no significant antigenic similarity with the integrin β chains such as CD18 (data not shown). Other possible related antigens include the ankyrin-associated gp90 (28) that appears to associate with the cytoskeleton as does ECMRIII (2). Similarly the cytoskeleton-associated protein described by Carraway and coworkers (29) as well as the hyaluronate receptor described by Underhill and coworkers (30) may be related. The intrinsic membrane glycosaminoglycans described by Bernfield and coworkers (31) and Hook *et al.* (32) as well as others also exhibit many characteristics common to the gp90 and the high molecular mass antigens described here, since both are highly sulfated, highly glycosylated membrane glycoproteins and both associate with the ECM.

It will be very interesting to determine the relation between the observed ligand-binding capability of some gp90s for collagen and the proposed biological function as a mediator of heterotypic cell–cell adhesion in different tissues. As well, the relative contributions of variability in primary amino acid core structure versus that conferred by posttranslational modification to ligand specificity have yet to be defined. The recent cloning of cDNAs encoding examples of these gp90s

(6, 33) should permit a more calculated approach to this interesting question.

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