Monoclonal antibodies reactive with NIH/3T3 cell surface antigens were obtained from hybridomas of murine myeloma cells fused to spleen cells of rats immunized with NIH/3T3 cell plasma membranes. Four of the antibodies, of forty that have been studied, appeared to react with allospecific antigens: they bound to NIH/3T3 cells but not to BALB/c/3T3 cells. Each of these four antibodies immunoprecipitated a glycoprotein of about 80,000 daltons that migrated to an isoelectric point of about pH 5.0. Polypeptides of identical molecular weight and isoelectric points, and yielding the same proteolytic cleavage fragments, were present in BALB/c/3T3 cells, but were not antigenically reactive. The 80,000-dalton glycoprotein was a major constituent of the plasma membrane. It was a predominant lactoperoxidase iodinated component of intact NIH/3T3 cells, and saturation binding of 125I-labeled antibody indicated that there were about 10^6 antigenic sites/cell. Studies of the distribution of the immunoreactive glycoprotein among different strains of mice confirmed the polymorphic expression of the determinant: Spleen cells of BALB/c, DBA/2, and CBA mice did not bind anti-80,000-dalton glycoprotein monoclonal antibodies, whereas spleen cells of a large number of other strains of mice were positive for antibody-binding. The antigenic reactivity varied markedly among different cell lines and was greatest with the NIH/3T3 mouse embryo fibroblast, G8-1 Swiss Webster myoblast, and IC-21 SV40-transformed C3H10T1/2 mouse peritoneal macrophage. The properties of the 80,000-dalton glycoprotein characterized this molecule as a new cell surface differentiation alloantigen of murine mesenchymal cells.

Alloantigens are properties of a number of cell surface proteins that are specified by polymorphic genes. All of the known proteins of the closely linked major histocompatibility complex genes are polymorphic; these include the H-2 (1), thymus leukemia (TL) (2), Qa (3), and immune response (Ia) (4, 5) alloantigens. Several lymphocyte cell surface differentiation proteins are alloantigens: the Thy-1 antigen (6), proteins of the Lyt series (7, 8), proteins of the Lyb series (9), Pca-1 (10), the T-200 (11, 12), and leucocyte common antigen (13, 14), and a recently described glycoprotein of about 100,000 daltons (15). Many murine cells also express on their cell surface the highly polymorphic proteins of endogenous retroviruses, particularly the viral glycoproteins of about 70,000 daltons (16).

The importance of these alloantigens is that they identify proteins that have been implicated in a number of cell recognition, receptor, and differentiation phenomena. For example, complex genetic polymorphism appears to be a fundamental property of the major histocompatibility system. Other alloantigens have been found primarily on lymphoid cells. The discovery of the Lyt-1 and Lyt-2 alloantigens, by Boyse and co-workers (2), pioneered the identification of numerous other polymorphic antigens that distinguish functional subpopulations of hematopoietic cells (7).

We previously have reported a collection of hybridoma monoclonal antibodies prepared by fusion of mouse myeloma cells and spleen cells of rats immunized with NIH/3T3 cells or plasma membranes (18). Hybridoma cell lines that secreted antibodies which bound to intact NIH/3T3 cells were selected and cloned. By use of these antibodies we have identified a number of previously uncharacterized cell surface proteins (18). The present report describes several distinctive features of one of these components, a glycoprotein of about 80,000 daltons. 1) It was a major component of the cell surface with about 10^6 sites/cell. 2) Four independent monoclonal antibodies that specifically precipitated this glycoprotein each reacted with an antigenic determinant that was polymorphic and was present only in cells and tissues of certain strains of mice. 3) The glycoprotein showed a unique pattern of cell expression; among a variety of cell lines of diverse strain and tissue origin, it was detected specifically on fibroblast, myoblast, and macrophage cell lines. The properties of the 80,000-dalton glycoprotein defined this molecule as a previously undescribed, allospecific, differentiation antigen of the murine cell surface.

**EXPERIMENTAL PROCEDURES**

**Animals**—All inbred strains of mice 6 to 10 weeks of age were obtained from Jackson Laboratories (Bar Harbor, ME) except for National Institutes of Health Swiss mice, which were from M. A. Bioproducts (Rockville, MD).

**Antibodies**—Hybridoma antibodies AMF-8 and AMF-12 were obtained from independent clones isolated from the same cell fusion experiment, as previously described (18). AMF-15 and AMF-16 hybridoma antibodies were obtained in a similar manner, but from two different cell fusion experiments. The sources of hybridoma antibodies were either the hybrid cell culture supernatants concentrated 20-fold by ammonium sulfate precipitation or ascites fluids from BALB/c nu/nu mice bearing hybrid cell tumors. Hybridoma antibody AMF-12 was purified from ascites fluids by affinity chromatography with Protein-A Sepharose (19). Purified goat IgG anti-rat IgG has been used as a control.

\* The abbreviations used are: IgG, immunoglobulin G; gp 80, 80,000-dalton glycoprotein; Lgp 100, 100,000-dalton glycoprotein.
described (18). Purified rabbit F(ab')2 anti-rat IgG was a gift of Dr. Alfonso Colombatti of the Johns Hopkins University School of Medicine. Antibodies were labeled with 125I by the method of Jensenius and Williams (20). Class-specific antisera against rat immunoglobulins were purchased from Miles Laboratories (Elkhard, IN).

Antibody-binding Assays—Trace binding assays were performed by incubation of 1 × 10^6 to 1 × 10^7 freshly prepared spleen cells with 20 ng of purified 125I-labeled AMF-12 antibody for 1 h at 0°C in 100 μl of Dulbecco’s phosphate-buffered saline containing 10 mg/ml of bovine serum albumin (final concentration). The cells were collected by centrifugation, washed three times, and the bound radioactivity was counted.

Saturation binding assays were performed as described by Williams et al. (21). NIH/3T3 cells (2.5 × 10^6) were incubated with 50 μl of concentrated hybrid cell culture supernatant for 1 h at 0°C and washed three times. The cells were then incubated with various amounts of 125I-labeled rabbit F(ab')2 anti-rat IgG antibody (5 to 8 μCi/μg) in 50 μl for 1 h at 0°C. After washing the cells, the radioactivity was counted.

The conditions for the preparation of cell extracts and the solid phase cell extract-binding assay have been described in detail (18).

Antibodies that reacted with cell surface antigens of NIH/3T3 or BALB/c 3T3 cells, glutaraldehyde-fixed cells, and cell extracts. Partial proteolytic peptide mapping was carried out as described by Cleveland et al. (30).

Two-dimensional Gel Electrophoresis—Two-dimensional gel electrophoresis was performed as described by O’Farrell (31). Cell extracts or immunoprecipitated cell proteins were suspended in 50 μl of O’Farrell buffer A (9.5 M urea, 2% (w/v) Nonidet P-40, 2% (w/v) Ampholines, and 5% (v/v) 2-mercaptoethanol). Following isoelectric focusing, the samples were analyzed by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis on 10% gel slabs (32).

**RESULTS**

Characterization of Monoclonal Antibodies that Bind to NIH/3T3 Fibroblasts but not to BALB/c 3T3 Fibroblasts—Antibodies that reacted with cell surface antigens of NIH/3T3 cells were generated by immunizing rats with a partially purified plasma membrane fraction from NIH/3T3 cells, as previously described (18). Spleen cells of the immune rats were fused with the murine myeloma cell line P3-NSI/1-Ag4-1. Hybridomas that produced antibodies which reacted with surface antigens of NIH/3T3 cells were selected and cloned.

Four stable cultures (AMF-8, AMF-12, AMF-15, and AMF-16), from a total of 40 that have been characterized, produced antibodies which bound to antigens of NIH/3T3 but not BALB/c 3T3 cells (Fig. 1). Antibody-binding curves with a constant saturating amount of monoclonal antibody and increasing amounts of NIH/3T3 cell extracts showed an antigen-dependent binding of each of the four monoclonal antibodies.2 In contrast, monoclonal antibody binding to BALB/c 3T3 cell extracts was not observed at any antigen concentration. As a control, another hybridoma antibody which bound to an unrelated antigen, a 90,000-dalton glycoprotein, was included in the same experiment; in this case the binding curves were the same with both cell lines. The specificity for NIH/3T3 cells was observed with all antigen preparations, including intact cells, glutaraldehyde-fixed cells, and cell extracts.

Each of these cloned hybridomas secreted a heavy chain of 52,000 daltons and a single light chain, as determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis of reduced immunoglobulins metabolically labeled with [35S]methionine. In each case, the apparent molecular weight of the light chains was different from that of the k-light chain of the P3-NSI/1-Ag4-1 cell line. Double diffusion analysis, using class-specific antisera, showed that two of the antibodies (AMF-12 and AMF-15) reacted only with anti-rat IgG antibody; and two (AMF-8 and AMF-16), with anti-rat IgG antibody (data not shown). There was conclusive evidence that each hybridoma clone represented a single, unique cell fusion event and was derived from a different antibody-forming cell. The four hybridomas were obtained from three independent experiments, i.e., from three different immunized rats and three different cell fusion experiments (AMF-8 and 12, AMF-15, AMF-16). In addition, although the AMF-8 and AMF-12 hybridomas were derived from the same hybridization experiment, the secreted antibodies were of different subclasses.
Identification of a 80,000-dalton Protein Antigen by Immunoprecipitation—The antigenic target of the monoclonal antibodies was determined by immunoprecipitation and two-dimensional gel electrophoresis of proteins from NIH/3T3 cells labeled externally with $^{125}$I. A polypeptide of 80,000 daltons and an isoelectric point of pH 5.0 was a major cell surface constituent of NIH/3T3 cells. The number of antigenic sites per cell was calculated to be $0.7 \times 10^6$, based upon the specific activity of the $^{125}$I-labeled F(ab')$_2$ antibodies, and a binding ratio of second to first antibody of 4:1 to 2:1 (33). The results indicated that the 80,000-dalton glycoprotein was a major cell surface constituent of NIH/3T3 cells.

![Image of two-dimensional gel pattern](image_url)

**Fig. 2.** Identification of gp80 in two-dimensional gel autoradiograms of NIH/3T3 and BALB/3T3 $^{125}$I-labeled cell surface proteins. Cell surface proteins were labeled with $^{125}$I, extracted with 0.5% Nonidet P-40 and resolved by two-dimensional gel electrophoresis as described under "Experimental Procedures." A, total $^{125}$I-labeled NIH/3T3 cell extract (6.7 $\times$ 10$^5$ cpm); B, $^{125}$I-labeled NIH/3T3 cell proteins immunoprecipitated with the AMF-16 antibody (5 $\times$ 10$^6$ cpm); C, total $^{125}$I-labeled BALB/3T3 cell extract (7.5 $\times$ 10$^6$ cpm). The arrows indicate those polypeptides identified as gp80, glycoproteins with an apparent molecular weight of about 80,000 and an isoelectric point of about 5.0. The selected region of the two-dimensional gel is shown by appropriate molecular weight and pH markers.

$^3$ G. Mengod, E. N. Hughes, and J. T. August, unpublished observations.

![Image of partial proteolytic peptide maps](image_url)

**Fig. 3.** Partial proteolytic peptide maps of $^{125}$I-labeled gp80 from NIH/3T3 and BALB/3T3 cells. $^{125}$I-Labeled polypeptides isolated by immunoprecipitation as previously described (18) were excised from one-dimensional gels and analyzed by partial proteolytic digest with papain before proteinase K digestion. The gp80 from NIH/3T3 cells was digested by incubation with A, 1.0 ng; B, 10 ng; or C, 750 ng of papain. The gp80 from BALB/3T3 cells was digested by incubation with D, 1.0 ng; E, 10 ng; or F, 750 ng of papain. The two-dimensional gel pattern of labeled proteins from NIH/3T3 cells was digested by incubation with a 1.0 ng of protease K digestion. The gp80 from NIH/3T3 cells was digested by incubation with A, 1.0 ng; B, 10 ng; or C, 750 ng of papain. The gp80 from BALB/3T3 cells was digested by incubation with D, 1.0 ng; E, 10 ng; or F, 750 ng of papain.
supernatants from the AMF-15 hybrid cell line

4B4.13 hybrid cell line
duplicate samples. bound radioactivity was counted. The results represent averages of incubated for 1 h at 0 °C with increasing amounts of 125I-labeled rabbit F(ab'Iz anti-rat IgG antibody (0.8 μCi/μg), washed, and the bound radioactivity was counted. The results represent averages of duplicate samples.

**TABLE 1**

**Antibody binding to spleen cells from different murine strains**

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Antibody bound (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td>NIH Swiss</td>
<td>1.9</td>
</tr>
<tr>
<td>C3H/HeJ</td>
<td>2.2</td>
</tr>
<tr>
<td>A/J</td>
<td>1.9</td>
</tr>
<tr>
<td>C58/J</td>
<td>1.7</td>
</tr>
<tr>
<td>C57BL/6/J</td>
<td>1.5</td>
</tr>
<tr>
<td>C57BR/cdJ</td>
<td>1.6</td>
</tr>
<tr>
<td>AKR/J</td>
<td>1.3</td>
</tr>
<tr>
<td>SWR/J</td>
<td>1.7</td>
</tr>
<tr>
<td>S/J/J</td>
<td>1.2</td>
</tr>
<tr>
<td>129/J</td>
<td>1.5</td>
</tr>
<tr>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>BALB/c/J</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>DBA/1/J</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>DBA/2/J</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>CBA/J</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

with each of the four anti-gp80 monoclonal antibodies.

**Antigen Expression in Different Cell Lines**—The effect of cell differentiation on the expression of the 80,000-dalton glycoprotein was analyzed by measuring antibody binding to extracts of 21 different cultured cell lines. Antigen-dependent binding of the anti-gp80 monoclonal antibodies was observed with extracts of NIH/3T3 fibroblast cells, NIH/3T3 cells transformed by Harvey sarcoma virus, G8-1 cells (Swiss Webster macrophages), and IC-21 cells (C57BL/6 SV40-transformed myoblasts) (Fig. 5). The antibodies did not react with extracts of 21 different cultured cell lines. Antigen-dependent binding of the anti-gp80 monoclonal antibodies was specific to certain types of cells. Saturation binding of anti-gp80 monoclonal antibodies to the surface of NIH/3T3 cells showed that there were 7 × 10^4 to 1.4 × 10^5 antigenic sites/cell. This concentration was comparable to that of Thy-1, the major cell surface glycoprotein of thymocytes, with 6 × 10^5 molecules/cell (34). In keeping with this finding, the 80,000-dalton glycoprotein was the most prominent 125I-labeled plasma membrane component of NIH/3T3 and BALB/3T3 cells, as distinguished by two-dimensional polyacrylamide gel electrophoresis. In addition, Bretscher et al. (35) have confirmed the plasma membrane localization of the antigen and have shown by immunoelectron microscopy with the AMF-12 antibody that the 80,000-dalton glycoprotein, as well as Thy-1, was uniformly distributed throughout the surface of NIH/3T3 cells, except for the coated pits. These data indicate that the 80,000-dalton glycoprotein, with its relatively large mass and high concentration, is one of the major protein components of the plasma membrane of the murine fibroblast.

Another remarkable property of the 80,000-dalton glycoprotein was its polymorphism. The antigenic determinants recognized by the monoclonal antibodies were present in tissues of several strains of mice, but were not detected in tissues of BALB/c, DBA/1, DBA/2, or CBA mice. This difference in antigen reactivity between the strains may be attributed to genetic polymorphism, rather than lack of gene expression, as a major iodinated protein of identical electrophoretic mobility, isoelectric point, and iodinated tryptic peptide composition was present in BALB/3T3 cells. Whether the serologically

**DISCUSSION**

A murine cell surface glycoprotein of about 80,000 daltons has been identified and studied by use of monoclonal antibodies. This glycoprotein was noteworthy in several respects. 1) It was one of the major constituents of the plasma membrane. 2) The determinants recognized by the monoclonal antibodies were allospecific. 3) This allospecific determinant acted as a predominant immunogen in a xenogenic host. 4) The expression of the glycoprotein was specific to certain types of cells. Saturation binding of anti-gp80 monoclonal antibodies to the surface of NIH/3T3 cells showed that there were 7 × 10^4 to 1.4 × 10^5 antigenic sites/cell. This concentration was comparable to that of Thy-1, the major cell surface glycoprotein of thymocytes, with 6 × 10^5 molecules/cell (34). In keeping with this finding, the 80,000-dalton glycoprotein was the most prominent 125I-labeled plasma membrane component of NIH/3T3 and BALB/3T3 cells, as distinguished by two-dimensional polyacrylamide gel electrophoresis. In addition, Bretscher et al. (35) have confirmed the plasma membrane localization of the antigen and have shown by immunoelectron microscopy with the AMF-12 antibody that the 80,000-dalton glycoprotein, as well as Thy-1, was uniformly distributed throughout the surface of NIH/3T3 cells, except for the coated pits. These data indicate that the 80,000-dalton glycoprotein, with its relatively large mass and high concentration, is one of the major protein components of the plasma membrane of the murine fibroblast.
detected differences between the 80,000-dalton glycoproteins are the result of variations in the carbohydrate and/or protein portion of the molecule remains to be determined. The 80,000-dalton glycoprotein appears to be different from all other murine cell surface alloantigens identified by polyclonal and monoclonal alloantisera. These include the Ala-1, H-2, Ia, Lyb series, Lyt series, Mls, Mph-1, Pca-1, Qa, and TLa alloantigens (for a review, see Ref. 9). The newly described Lgp100 (15), H9/25 (36), and Ly-10.1 (37) alloantigens identified by monoclonal antibodies can also be excluded. None of the above other alloantigens showed the same distribution of antigen expression among different strains of mice as did the gp80 and none has been assigned to a molecular species of 80,000 daltons.

The finding of four xenogeneic monoclonal antibodies, each of which reacted with an alloantigenic determinant of the same protein, was unexpected. Xenogeneic immunizations usually elicit responses to a wide range of antigenic determinants because of the evolutionary changes that occur in many proteins (33). It is for this reason that alloantigenic antisera have been prepared by selective immunization between different inbred mice and therefore were raised against a restricted population of antigenic loci (6). Thus, the high frequency of response to a polymorphic determinant of the 80,000-dalton glycoprotein was remarkable. The four hybridoma cell lines that produced antibodies reactive with the gp80 were present among a total of 40 cloned hybridomas that have been characterized. This frequency was exceeded only by hybridomas secreting antibodies to a 90,000-dalton protein, for which there were six independent isolates. As the four monoclonal antibodies of IgG1 and IgG2 subclasses were obtained from spleenocytes of three different immunized rats and three separate cell fusion experiments, the multiplicity of these anti-gp80 hybrid clones cannot be attributed to activation and division of a single antibody-forming precursor cell. Moreover, studies in progress suggested that each of the four antibodies acted on the same or proximate alloantigenic determinant. This is to be compared with the reported xenogeneic, monoclonal rat antibodies against the Lyt-1, Lyt-2, Thy-1, and T-200 molecules, which reacted with framework determinants as well as the alloantigenic determinants of the corresponding polymorphic molecules (38). The glycoprotein is further emphasized by the relatively uncommon detection of alloantigens by xenogeneic monoclonal antibodies. In continuing studies in this laboratory, among more than 40 monoclonal antibodies that have been tested, alloantigenic specificity was observed only with the anti-gp80 antibodies. Springer et al. (39) tested 10 rat anti-murine lymphocyte monoclonal antibodies against a large number of murine strains; no antigenic polymorphism was detected.

The presence of an alloantigenic immunodominant antigenic site on the 80,000-dalton glycoprotein suggested that this protein might also characterize cellular differentiation. The remarkable finding was that the antigenic determinant reactive with the anti-gp80 monoclonal antibodies was present on fibroblast lines from Swiss mice and on a macrophage cell line from C57BL/6 mice, but it was not detected on a C57BL/6 T-cell lymphoma, a C57BL/6 lung carcinoma, an A/J neuroblastoma, or a 129/J embryonal carcinoma. In other studies, we have also found that the 80,000-dalton glycoprotein was a major differentiation antigen of hematopoietic tissues. Radioimmune precipitation and fluorescence-activated cell-sorting analyses showed that the glycoprotein antigen characterized the phagocytic cell lineage, as it was present on macrophages, granulocytes, monocytes, and many adult bone marrow cells but was absent on other lymphoid cells. Thus, among the cells tested, the expression of the glycoprotein was restricted to certain types of mesenchymal cells.

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