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Immunological Analysis of a Glycoprotein (Contact Sites A) Involved in Intercellular Adhesion of Dictyostelium discoideum

Ben A. Murray, Lisa D. Yee, and William F. Loomis

Department of Biology, University of California at San Diego, La Jolla, California 92093

We have prepared antisera in rabbits to the "contact sites A" glycoprotein (gp80) purified from Dictyostelium discoideum. IgG isolated from these antisera reacts with a number of different proteins in D discoideum lysates, as analyzed by immune precipitation and by antibody staining of gel electropherograms transferred to nitrocellulose. Blocking experiments indicate that this cross-reactivity reflects the presence of common antigeneic determinants on gp80 and other cellular proteins, rather than the presence of extraneous antibodies in the antisera. The spectrum of reactive proteins is different at different stages of development. In particular, gp80 itself is synthesized only for a restricted period during the cell aggregation phase. The protein persists throughout development and can be detected in spores. Anti-gp80 Fab fragments bind to the surface of developing D discoideum cells and specifically block their developmentally regulated adhesion. After absorption with vegetative cells, the IgG stains only gp80 and (to a lesser extent) one other band in lysates of aggregation-competent cells. The absorbed antibodies also can block adhesion. Several proteins that appear late in development also are stained by the absorbed IgG.

Key words: adhesion, Dictyostelium discoideum, contact sites A, development, immune staining after polyacrylamide gel electrophoresis

During the starvation-induced development of the cellular slime mold Dictyostelium discoideum, the cells develop the ability to form strong EDTA-resistant intercellular adhesions [1]. In an elegant series of investigations, Beug et al [2,3] have demonstrated that these adhesions can be abrogated by specific Fab antibody fragments directed against new antigenic sites ("contact sites A") that appear on the surfaces of the developing cells. More recently, Müller et al [4,5] have shown that the relevant target of these Fab fragments is apparently a single glycoprotein that can be purified from membranes isolated from developing cells. This glycoprotein ("gp80") has an apparent molecular weight of 80,000 as measured by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and contains approximately 25% carbohydrate [5].

In separate investigations, researchers from several laboratories have suggested that the developmentally regulated galactose-binding lectin discoidin I may

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also play an essential role in cell–cell adhesion [6–8]. The mutant strain HJR1 produces normal amounts of discoidin I, which has lost its ability to bind to galactose, and this strain does not develop the ability to form EDTA-resistant adhesions [7,8]. However, it is possible that the mutation in strain HJR1 may block the dependent sequence of events [9] required for the development of EDTA-resistant adhesions; indeed, Marin et al [10] have suggested that discoidin forms a part of one of two systems of intercellular communication that are required for this process to occur. The relationship, if any, between discoidin I and gp80 remains unclear, although several lines of evidence [4,11,12; J. Ray and R. Lerner, unpublished results] suggest that gp80 does not act as a receptor for the sugar-binding activity of discoidin I.

Although a considerable amount is known about the molecular structure and developmental regulation of discoidin [13–17], comparatively little is known of the corresponding properties of gp80. Here we report the raising and characterization of a rabbit antiserum directed against purified gp80. Using this antiserum, we show that the synthesis of gp80 is restricted during development to the period of cell aggregation, although the accumulated protein persists throughout the remainder of development and can be detected in spores. We also show that several other developmentally regulated proteins of D discoideum cross-react immunologically with gp80.

METHODS

Cell Strains, Growth and Development

All experiments were performed at least twice. Strains HL100 (a spontaneous mutant of strain NC4, which lacks the ability to grow on Bacillus subtilis [18]) and AX3 [19] were grown in association with Klebsiella aerogenes at 22°C as previously described [20]. AX3 also was grown axenically in HL5 medium [21]. Conditions for cell harvesting, development on Millipore filters, and labeling with ^{35}S-methionine (New England Nuclear Corp., Boston, MA) have been described [22]. Generally 5×10^7 cells were developed on a 4.7 cm-diameter filter and were labeled with 50 μCi of ^{35}S-methionine for 2 h.

Purification of gp80

Gp80 was prepared by the method of Müller et al [5] from 5 to 10×10^{10} axenically-grown AX3 cells. The purification was monitored by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) [23]. Yields were comparable to those of Müller et al [5]. In our hands, the recovery of gp80 after the final sucrose gradient step [5] was poor, so we replaced this with a preparative isoelectric focusing step in a flat bed of polyacrylamide beads (BioRad Laboratories, Richmond, CA) [24] containing 0.1% (v/v) Triton X-100 plus a 2% (w/v) solution of a 70:30 mix of pH 3–7 and pH 4–9 technical-grade ampholytes (BioRad Laboratories). The bed was focused for 47,500 V-h with the final 22 h being run at 1,000 V. Fractions containing gp80 (assayed by SDS-PAGE) were pooled, concentrated, and dialyzed extensively against 10 mM Tris/HCl, 0.5 mg/ml sodium cholate, pH 7.5 [5]. Small aliquots were stored frozen at −70°C. Protein was assayed according to Bradford [47] using a commercial reagent (BioRad).

Preparation of Antisera and IgG

Antisera against gp80 were raised in rabbits using a modification of the method of Goudie et al [25]. Two rabbits were immunized with 20 μg of electrophoretically purified gp80 (method B of Müller et al [5]), emulsified in Freund's complete adjuvant and divided among the popliteal lymph nodes and 10 intradermal sites along the back. The rabbits were boosted twice (5 weeks and 11 weeks after the initial injection) with 20 μg of sucrose-gradient-purified gp80 (method A of Müller et al [5]), emulsified in Freund's incomplete adjuvant and divided between subcutaneous sites on the back and intramuscular sites in the hind legs. The rabbits were exsanguinated 10 days after the second boost. IgG was prepared from these sera by chromatography on protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) [26]. Fab fragments were prepared as previously described [2], except that Fab and Fc fragments were separated by passage over protein A-Sepharose. The purity of the IgG and Fab preparations was verified by SDS-PAGE.

Where indicated, IgG or serum was preabsorbed three times by incubation for 2 h on ice with vegetative AX3 cells (2.5×10^8 cells per 5 mg IgG or 1 ml serum for each absorption).

Rabbit antiserum against discoidin [14,17] has been described previously.

Polyacrylamide Gel Analysis

Two-dimensional gel analysis was by the method of Garrels [27]. SDS-PAGE was carried out using the system of Laemmli [23]. Gels were stained with Coomassie brilliant blue R-250 or, more recently, with the simplified silver stain [28] as modified in this laboratory (J. Morrissey, Anal Biochem, in press). Periodic acid-Schiff staining was by published methods [29,30].

Electrophoretically separated proteins were transferred to nitrocellulose filters and stained with antiserum or IgG according to the method of Towbin et al [31] with the following modifications: soaking solutions contained 30 mg/ml crude ovalbumin (Sigma Chemical Co., St. Louis, MO) instead of bovine serum albumin; the soaking solutions were clarified by centrifugation at 12,000g for 10 min before use. All immunological staining solutions contained 0.1% (v/v) Triton X-100 (BioRad Laboratories). Bound antibody was detected using ^{125}I-labeled staphylococcal protein A [32] (Pharmacia Fine Chemicals). About 1 μCi (37 kBq) of iodinated material was used for each gel. Gels were exposed to preflashed [33] Kodak X-Omat R film with a Dupont Cronex Lighting-Plus intensifying screen at $-70°$C to prepare autoradiograms [34].

Immune Precipitation

Cell lysates (10^7 cell equivalents/ml) in RIPA buffer (10 mM Tris/HCl, pH 7.2, 1% (v/v) Triton X-100, 10 mg/ml sodium deoxycholate, 1 mg/ml SDS, 0.14 M NaCl, 0.3 M D-galactose, 0.2 mg/ml NaN_3, 2 mM methionine, 1 mg/ml ovalbumin) [17] were centrifuged for 2 min at 13,000g to remove particulate material and were then incubated on ice for 2 h with the indicated amount of antiserum or IgG. Prewashed Staphylococcus aureus ("Pansorbin," Calbiochem-Behring Corp., La Jolla, CA) [35,36] was added, and the mixture was kept on ice a further 20–30 minutes. After 3–5 washes with RIPA buffer (the last 2 washes with buffer not containing ovalbumin), the well-packed bacterial pellet was ex-

Fig. 1. Gel electrophoretic analysis of gp80. Purified gp80 (80 ng) was analyzed by isoelectric focusing using pH 3.5–10 ampholytes (LKB Instruments Inc., Rockville, MD) followed by SDS-PAGE in a 10% polyacrylamide gel. The acid end of the focusing gel is to the left. Standards were run in separate wells on either side of the two-dimensional separation: gp80, 80 ng of purified gp80; MW, 20 ng each of myosin (molecular weight 200,000), β-galactosidase (116,000), conalbumin (84,000), bovine serum albumin (68,000), ovalbumin (45,000), and discoidin I (32,000). Proteins were visualized using the silver stain. Bands marked "A" are artifacts that are also seen when sample buffer without protein is run (J. Morrissey, Anal Biochem, in press).

tracted at 95°C for 3 min with 25 μl sample buffer [23] and analyzed by SDS-PAGE as described above. Radioactive proteins were visualized by fluorography [37] using "Enhance" (New England Nuclear Corp.)

RESULTS

Preparation and Characterization of gp80 and Anti-gp80

A silver-stained two-dimensional electropherogram of the purified gp80 is shown in Figure 1. We identified this material as the "contact sites A" protein of Müller et al [4,5] by several criteria: 1) The protein partitions during the purification in the same way as does the "contact sites A" glycoprotein. 2) The apparent molecular weight of 84,000 daltons is consistent with that reported [4]. It should be noted that the exact apparent molecular weight of this protein varies depending on the porosity of gel used [5]. This phenomenon is observed frequently on polyacrylamide gel electrophoresis of glycoproteins. 3) The protein is stained strongly by the periodic acid-Schiff procedure for carbohydrate on both one- and two-dimensional polyacrylamide gels. 4) The protein binds to insolubilized concanavalin A, from which it can be (partially) released by α-methylmannoside. 5) In our hands, suspension-developed AX3 cells that have not been pulsed with cyclic AMP do not develop EDTA-resistant contacts ("contact sites A"). The protein cannot be purified from such cells. 6) Fab fragments and whole antibodies raised against the purified protein specifically block the EDTA-resistant adhesion of aggregation-competent cells (see below).

TABLE I. Blocking of Adhesion by Anti-gp80 Fab*

	Single and double cells (% of total)	
Blocking protein	−Fab	+Fab
None	49	89
Gp80 (30 μg/ml)	36	33

*Aggregation-competent HL100 cells were tested [45] in the presence or absence of 1.61 mg/ml anti-gp80 Fab plus the indicated blocking proteins. All assay solutions contained 10 mM EDTA. Cells were counted using a hemocytometer. Each entry is the mean from duplicate samples. The assay measures disappearance of single and double cells into larger aggregates, so lower values correspond to increased adhesion. Control experiments showed that equivalent dilutions of the gp80 storage buffer had no effect on adhesion or its blockade by Fab (not shown).

After two-dimensional gel electrophoresis, the purified protein appears as a set of four to seven equally spaced spots with the same apparent molecular weight but with slightly different isoelectric points (Fig. 1). The mean isoelectric point of the protein is approximately 3.5.

Purified gp80 runs as a single band with an apparent molecular weight of approximately 100,000 when analyzed under nonreducing conditions in a 5–15% linear acrylamide gradient gel (data not shown). Thus, reduction seems to alter the electrophoretic properties of the molecule, but there is no evidence for disulfide-mediated crosslinking of different gp80 molecules to form multimeric structures.

Antisera against the purified gp80 were raised in rabbits as described in "Methods" and IgG was prepared by affinity chromatography on protein A-Sepharose. Fab fragments prepared from this IgG are capable of blocking EDTA-resistant adhesion (Table I). This property provided one of the original criteria for the definition of contact sites A [2]. Preabsorption of the Fab fragments with purified gp80 abolished their ability to block adhesion (Table I). The implication that the antibodies recognize cell-surface determinants was verified by direct binding studies (data not shown), which showed that 1) anti-gp80 IgG binds specifically to the surface of both developed and vegetative cells; 2) substantially more IgG is bound per cell to the developed than to the vegetative cells; and 3) all specific binding to both cell types is abolished by preabsorption of the IgG with small amounts of purified gp80.

In addition to reacting with gp80, this IgG also reacts specifically with numerous proteins present in vegetative cells, as assayed by immune precipitation of ^{35}S-methionine-labeled proteins (data not shown) or by immunostaining of electropherograms of whole cell extracts after transfer to nitrocellulose (Fig. 2). After three rounds of absorption with vegetative AX3 cells, most of the vegetative-cell staining is abolished, while strong staining for gp80 is retained. Purified gp80 blocks all staining by unabsorbed or absorbed IgG (Fig. 2), suggesting that the staining of the vegetative species results from the presence of crossreacting determinants on these species and gp80, rather than from the presence of irrelevant anti-vegetative-cell-protein antibodies in the IgGs. Preimmune serum or IgG do not stain any bands.

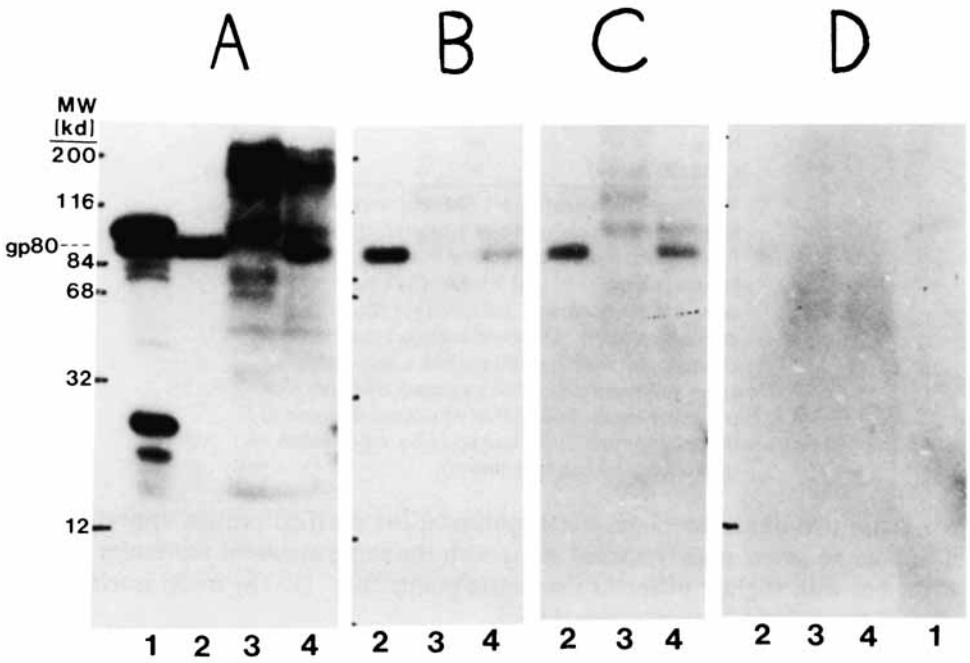

Fig 2 Absorption of anti-gp80 IgG Extracts from 10^5 HL100 cells or spores were electrophoresed on 5–15% acrylamide gradient gels and transferred to nitrocellulose filters After staining with Amido black to visualize molecular weight markers (Fig 1, migration positions are marked to the left of panel A), the filters were cut apart and stained with IgG (0 4 μg/ml) Antibody-binding bands were detected by ^{125}I-protein A binding followed by autoradiography Samples were HL100 spore extract (well 1), purified gp80 plus molecular weight standards (well 2), vegetative HL100 cell extract (well 3), or extract from HL100 cells after 7 5 h of development on Millipore filters (well 4) Panel A was stained with unabsorbed anti-gp80 IgG, panel B, with IgG absorbed with vegetative AX3 cells, panel C, with IgG absorbed with a detergent (RIPA buffer) lysate of vegetative AX3 cells, and panel D, with IgG absorbed with 1 μg/ml purified gp80 (the same material analyzed in well 2) Gp80 at 0 1 μg/ml was not effective in absorbing antibody staining ability for any of the major bands in any sample (data not shown)

We used the assay of Springer and Barondes [38] to verify that the absorption with vegetative cells does not abolish the ability of the antibodies to block EDTA-resistant adhesion. This assay employs intact anti-gp80 IgG or serum with the addition of Fab fragments of goat anti-rabbit IgG to prevent antibody-mediated cell agglutination. The blockage of adhesion using this assay was not as complete as that caused by the Fab fragments directed against anti-gp80, but it is clear that the absorbed serum is capable of blocking adhesion to the same extent as is the unabsorbed serum (Table II). We have not determined whether those antibody species removed by the absorption would also be capable of blocking adhesion.

Accumulation of gp80 and Discoidin

To determine the pattern of gp80 accumulation during development, we analyzed extracts of whole cells of D discoideum that had been allowed to develop on Millipore filters for varying lengths of time. The separated proteins were transferred to nitrocellulose, stained with antiserum or purified IgG, and

TABLE II. Blocking of Adhesion by Absorbed Anti-gp80 IgG*

	Single-cells (% of total ± sem)	
Antiserum	−gp80	+gp80
None	50 ± 4	ND
Preimmune	54 ± 3	51 ± 3
Anti-gp80 (unabsorbed)	68 ± 4	ND
Anti-gp80 (absorbed)	72 ± 3	54 ± 4

*Cell–cell adhesion was assayed by the method of Springer and Barondes [38] using the indicated rabbit sera diluted 1:100 plus Fab fragments of goat anti-rabbit IgG (Cappell Laboratories, Cochranville, PA) at 0.25 mg/ml. Where indicated, rabbit antiserum against gp80 was exhaustively absorbed with vegetative AX3 cells before use. All assay solutions contained 10 mM EDTA. Where indicated, purified gp80 at 8 μg/ml was incubated with the sera before use in the assay. Loss of single cells into larger clumps was measured using an electronic particle counter, so lower values correspond to increased adhesion. Each entry gives the mean ± sem of six determinations from two experiments. ND, not determined.

visualized using ^{125}I-protein A. Figure 3A shows that gp80 can be detected in bacterially grown cells of strain AX3 at about 10 h of development and persists throughout the remainder of development. Gp80 is first detected some 6 h earlier during development of strain AX3 which was grown axenically in HL5 medium (Fig. 3B). The protein can be detected also in well-washed spores (Figs. 2 and 4). In bacterially grown HL100 cells, gp80 can first be detected 1–2 h earlier than bacterially grown AX3, consistent with the slightly slower overall development displayed by strain AX3 (data not shown).

We also examined the accumulation of discoidin I in the same experiments (data not shown). In all cases, discoidin can be detected from 0 to 4 h in advance of gp80 and persists throughout the remainder of development. In particular, and in agreement with the earlier work of others, discoidin can be detected in AX3 cells growing axenically in HL5 [39]. Gp80, in constrast, cannot (Fig. 3).

The 84,000 dalton band corresponding to gp80 is not the only band stained by the anti-gp80 antibodies in these gels. A band with an apparent molecular weight of 95,000 daltons (p95) always appears coordinately with gp80 (Fig. 3). This band is not detectable in the purified gp80 preparation, yet all staining of the 95K band can be abolished by preabsorption of the anti-gp80 IgG with purified gp80 (Fig. 2). This argues strongly that the p95 and gp80 bands share antigenic determinants. At 14–16 h of development (at the time of slug formation), another band with an apparent molecular weight of about 25,000 daltons appears (p25) (Fig. 3). Finally, at very late times of development, and in spores, there can be detected an additional band of molecular weight ~ 90,000, which cannot be detected at earlier stages (Fig. 2). In all cases, staining of these bands is abolished by preabsorption of the IgG with purified gp80 at approximately the same rate as the staining of gp80 itself is abolished (Fig. 2), arguing that these species, too, share antigenic determinants with gp80.

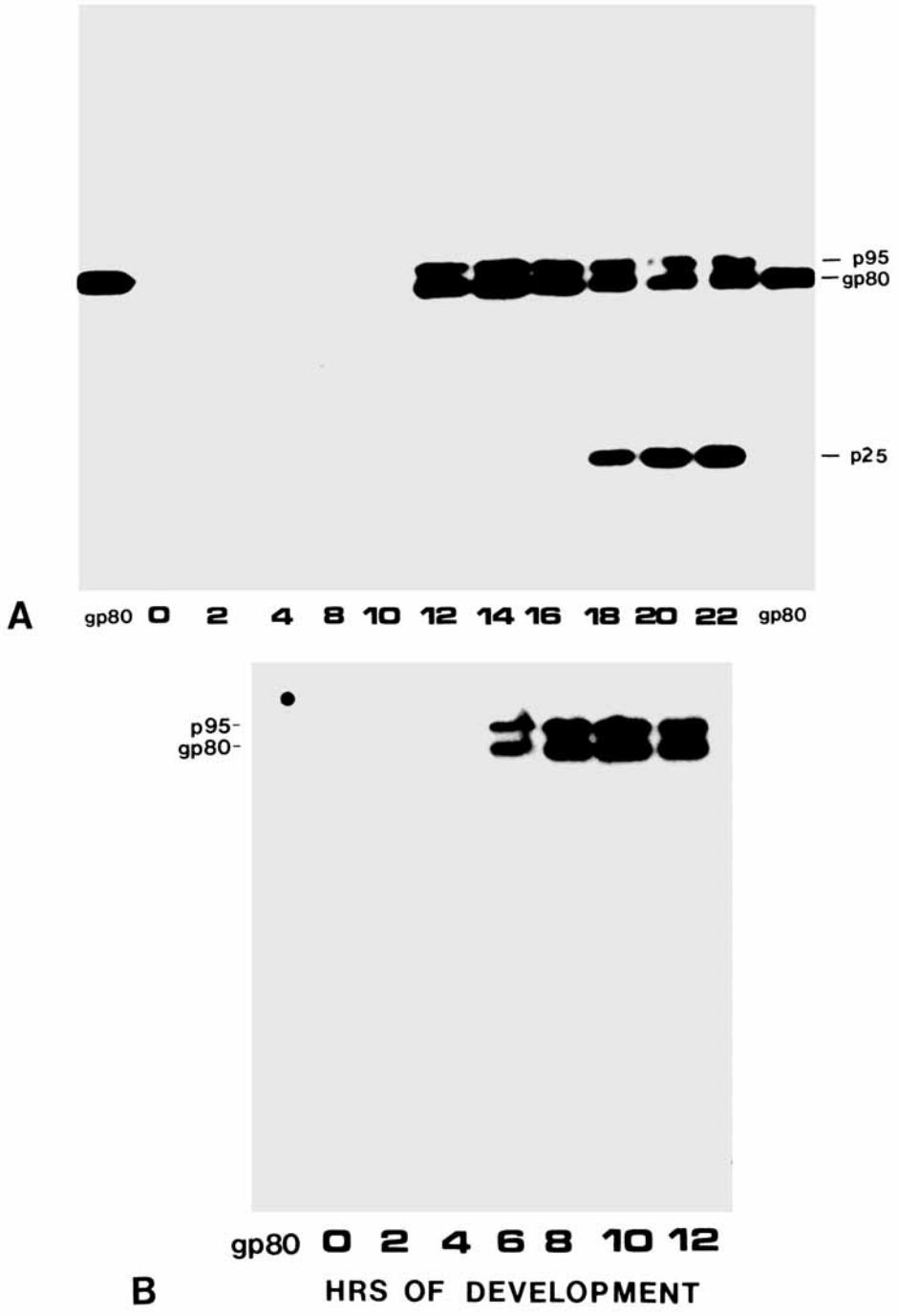

Fig. 3. Accumulation of gp80. Bacterially grown AX3 cells (panel A) or axenically grown AX3 cells (panel B) were starved for varying lengths of time on Millipore filter pads, collected, washed, and dissolved at 10^7 cells/ml in SDS-PAGE sample buffer [23]. Aliquots corresponding to 10^5 cells were electrophoresed on 8% acrylamide gels, transferred to nitrocellulose, and stained with anti-gp80 IgG that had been preabsorbed with vegetative AX3 cells. Numbers indicate starvation time (h) before cells were collected; 0 refers to vegetative cells. Wells marked "gp80" contained purified gp80.

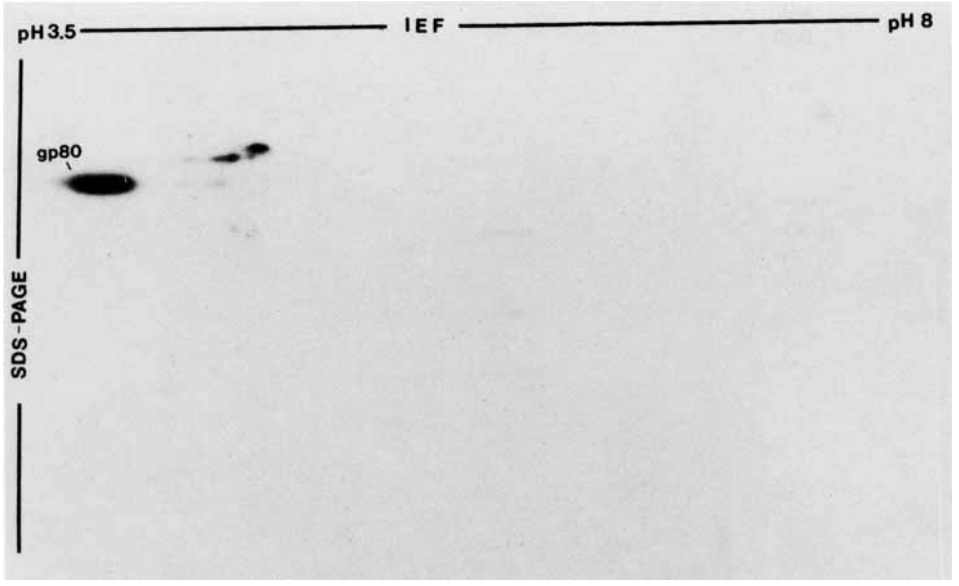

Fig. 4. Presence of gp80 in mature spores. Extracts of HL100 spores (prepared by grinding in liquid N_2) were analyzed as described in Figure 1, but using a 10% gel for the second dimension. Proteins were transferred to nitrocellulose and stained with anti-gp80 serum.

In general, migrating slugs are not formed during development on Millipore filters under our conditions. In several experiments whole migrating slugs or front and back segments of migrating slugs were collected, and extracts were analyzed by the nitrocellulose filter-transfer method. Gp80, p95 and p25 all were clearly detected in all of the slug samples tested (data not shown).

Synthesis of gp80 and Discoidin

To examine the pattern of synthesis of gp80 and discoidin during development, cells were pulse-labeled for 2 h periods with ^{35}S-methionine, lysed in detergent-containing buffer, and precipitated using anti-gp80 or anti-discoidin. The results were consistent with the accumulation studies. Synthesis of gp80 was maximal in bacterially grown HL100 cells between 6–8 h of development (data not shown), in bacterially grown AX3 cells between 8 and 10 h of development (Fig. 5), and in axenically grown AX3 cells between 3 and 6 h of development (data not shown). In more heavily exposed autoradiograms p95 showed the same kinetics of synthesis as gp80. Discoidin synthesis peaked btween 0 and 2 h before gp80 synthesis in each case. Discoidin synthesis was detectable in AX3 labeled during vegetative growth in HL5, while gp80 synthesis was not (data not shown). Synthesis of both gp80 and discoidin is restricted to limited time periods during development; for both proteins, synthesis ceases by about 14 h (for bacterially grown cells) (Fig. 5) or 10 h (for axenically grown cells) (data not shown).

Late in development, the anti-gp80 antibodies precipitate a number of strongly labeled proteins (Fig. 6). With one exception (p25), the same proteins are precipitated by an antiserum directed against SDS-PAGE-purified spore coat proteins (Loomis, unpublished). The apparent molecular weights of the precipitated proteins also agree well with those of the spore coat proteins studied by Orlowski

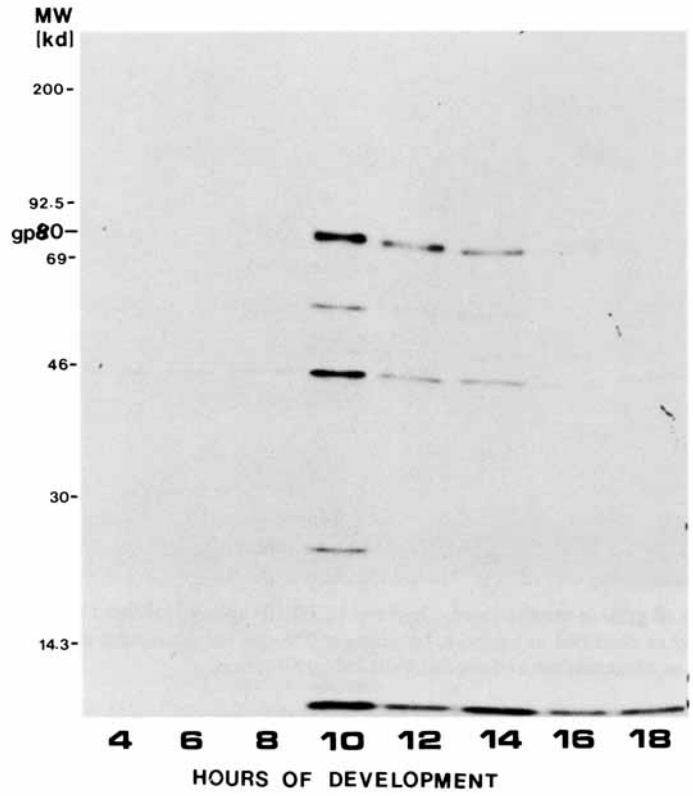

Fig. 5. Synthesis of gp80. Bacterially grown AX3 cells were developed on filters for the indicated times and were pulsed with ^{35}S-methionine for the final 2 h. Cells were analyzed by immune precipitation using vegetative-cell-absorbed anti-gp80 IgG as described in the legend to Figure 6. Bands other than gp80 are nonspecifically precipitated bands that were also seen when preimmune IgG was used instead of anti-gp80 IgG (data not shown).

and Loomis [40]. With the possible exception of a 95,000 dalton band, these proteins are not stained by the anti-gp80 antibodies on nitrocellulose transfers (Figs. 2, 3, and 5), and conversely the anti-spore coat serum neither precipitates ^{35}S-methionine-labeled gp80 nor stains gp80 on nitrocellulose transfers (data not shown).

There is one protein of approximately 25,000 daltons molecular weight (p25) that is precipitated by the anti-gp80 antibodies (Fig. 5) but not by the anti-spore coat serum. The time of synthesis and apparent molecular weight of this protein suggests that it corresponds to the same 25,000 dalton band detected by anti-gp80 on nitrocellulose filter transfers (Figs. 2 and 3).

Persistence of gp80 in Cells and Membranes

Parish and co-workers [41,42] have reported that the gp80 protein is lost from the membranes of cells late in development. Since our filter-transfer results indicated that, to the contrary, gp80 persists throughout development and is even found in spores, we decided to verify the persistence of the protein by an additional method and to examine directly whether the protein persists in the cell

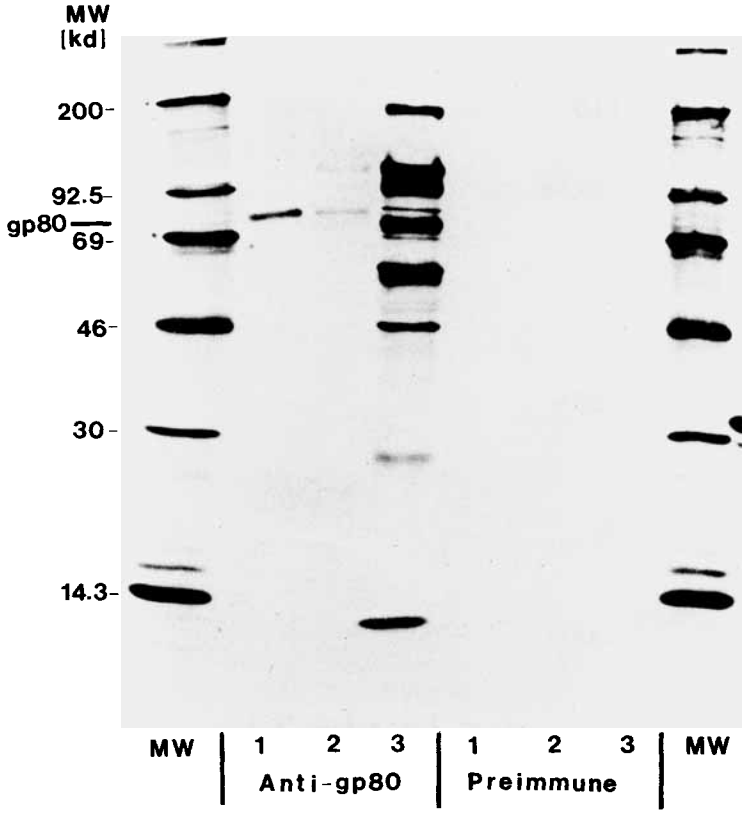

Fig. 6. Pulse-chase analysis of gp80. HL100 cells were pulse-labeled with ^{35}S-methionine from 6 to 8 h of development and were collected immediately (well 1) or were transferred to fresh filters containing nonradioactive methionine and collected at 22 h of development (well 2). Other cells were pulse-labeled from 20 to 22 h of development and were collected immediately (well 3). Cells were washed, lysed in detergent-containing buffer, and immune precipitated (2.5×10^6 cells per sample) either with anti-gp80 IgG (preabsorbed with vegetative cells) or with preimmune IgG as indicated. Wells labeled "MW" contain ^{14}C-labeled molecular weight standards as indicated to the left of the figure. Samples were analyzed by SDS-PAGE on a 5–15% gel followed by fluorography.

membrane. The immune precipitation results in Figure 6 demonstrate that at least a portion of the gp80 that is synthesized at 6–8 h of development remains in the cell at 20–22 h, a time when no new gp80 is being synthesized. Immune staining of nitrocellulose filters (Fig. 7) also demonstrates that gp80 remains in the membranes even at 22 h of development. Other samples run on the same gel (not shown) demonstrated that the filter-transfer technique is linearly sensitive to changes in the amount of gp80 present in the sample in the range of protein analyzed on this gel. By cutting out the nitrocellulose strips corresponding to gp80 and counting them in a liquid scintillation counter, we estimated that about 85% of the gp80 in the 22 h samples resides in the membrane, whereas about 90% resides in the membrane in the 10 h samples.

Twenty-two-hour cells or membranes contained 45–50% of the level of gp80 found in their 10 h counterparts. In the same experiment, the amount of protein per cell at 22 h was 53% of the amount at 10 h. Thus, gp80 seems to decay at a rate similar to that of total cellular protein.

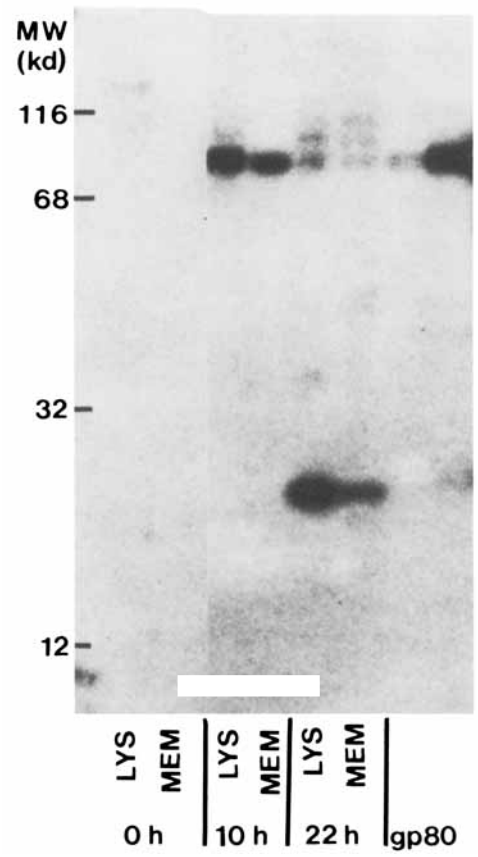

Fig 7 Analysis of gp80 in membranes of developing cells Membranes were purified from digitonin-lysed cells by a two-phase polymer method [14] and were analyzed on a 10% gel ("MEM") along with whole-cell lysates ("LYS", 10^5 cells per sample) Membrane samples were normalized to 10^5 cell equivalents per sample using alkaline phosphatase as a membrane marker [14,46] The rightmost wells contained 1 and 10 ng of purified gp80, respectively Proteins were transferred to nitrocellulose and stained with anti-gp80 IgG that had been preabsorbed with vegetative cells Samples were from HL100 cells at 0 h, 10 h, or 22 h of development

Figure 7 also shows that p25 can be found in the membrane, whereas p95 cannot. From this and other experiments, we estimate that at most 10% (and perhaps much less) of the p95 in the cell is present in the membrane.

DISCUSSION

The power of functional analyses using complex antisera has been beautifully demonstrated by Gerisch and his co-workers in the study of "contact sites A" [1-5]. Indeed, to date this has been one of the few approaches that has successfully provided direct evidence for the involvement of specific molecules in the developmentally regulated adhesive system of D discoideum. A full understanding of the molecular mechanism of this adhesive system and of the mechanistic role within this system, whether direct or indirect, of molecules such as gp80 will be facilitated by the availability of specific probes for such molecules.

The significance of the acidic isoelectric point and charge heterogeneity of gp80 cannot yet be assessed. It seems unlikely that the heterogeneity is simply a result of degradation during the purification procedure, since it is also seen when immune precipitates from pulse-labeled cells are analyzed by two-dimensional gel electrophoresis (data not shown).

It should be noted that neither we (Fig. 1) nor Müller et al [4,5] have purified the protein to homogeneity. However, the results shown in Figure 2 demonstrate that the contaminating species are not detected by the anti-gp80 antibodies. We should be able to detect (on this and longer exposures of the same gel) bands binding less than 1% of the amount of ^{125}I-protein A bound by the gp80 band. Some preparations (for example, see Fig. 7) do contain other material that reacts weakly with the anti-gp80 antiserum; however, even preparations in which this material is undetectable (Fig. 2) are fully capable of blocking staining of all reactive bands in the electropherogram. Furthermore, approximately equivalent amounts of absorbing material are required to block the staining of gp80 and of the crossreacting bands, even though (by immune staining) any contaminants in the absorbing preparation of gp80 are at least 2 orders of magnitude less immunoreactive than was the gp80 itself. From these considerations, we feel that it is reasonable to conclude that the data reflect a true crossreactivity between gp80 and other protein species of D discoideum rather than the presence of antibodies directed against contaminants in the immunogen.

Using the antibodies described in this paper, we have shown that gp80 molecules appear, by de novo synthesis, with kinetics that parallel the appearance of EDTA-resistant "contact sites A" on developing cells [1–5]. Growth of cells in axenic medium results in the simultaneous acceleration of the appearance of gp80 and EDTA-resistant adhesion. Our results for discoidin synthesis and accumulation confirm previous reports [13–15,39].

The developmental regulations of gp80 and of discoidin I show several similarities. Both are synthesized only during a restricted period of time during development, both are accelerated in their time of appearance in axenically grown cells, and once synthesized both persist throughout development. However, discoidin seems to appear slightly in advance of gp80 in bacterially grown cells. Discoidin definitely appears before gp80 in axenically grown cells, since discoidin is present in vegetative axenically grown cells, and gp80 is not. The possibility then arises that the expression of gp80 may depend on the previous expression of discoidin, and thus that the adhesion-blocking effect of the HJR1 mutation [7] may reflect a pleiotropic effect of this mutation on gp80 synthesis or expression. As yet there are no data bearing on this possibility.

Our finding that gp80 persists in the cell membranes throughout development conflicts with the results of Parish and co-workers [41,42]. These results were based on long-term labeling and immunological staining experiments. The long-term labeling results may be a result of the limited period of synthesis of gp80 during development and of the fact that cells incorporate about 10 times as much label (in our case ^{35}S-methionine) during culmination (18 h of development and after) as during earlier stages (data not shown). Thus, the label incorporated into gp80 early in development will be greatly diluted by label incorporated later. The immunological studies of Parish et al are difficult to interpret, but it appears that membrane preparations from different times during development were

reacted only against homologous antisera [42]. Rabbits can respond in complex manners to such complicated mixtures of antigens, and thus it is difficult to interpret such comparisons using different sera for each stage of development.

Several recent studies [43,44] have suggested that the adhesive system involving "contact sites A" is removed from the cells following aggregation and is replaced by a new (perhaps histiotypic) system. Our results suggest that such a replacement, if it affects gp80 at all, must act through a subtle serological or physiological alteration of the molecule rather than simply by removal of the molecule from the cell membrane or from the cells altogether.

Finally, the crossreactivity we observe between gp80 and several other molecules synthesized at several different times of development was unexpected. A common antigen shared by a number of D discoideum lysosomal enzymes has been described [48,49], but immune staining experiments using antiserum that recognizes this common antigen (kindly supplied by R. Dimond) show that it is not present in gp80 (unpublished results). At the time at which EDTA-resistant "contact sites A" and their sensitivity to immune blockade are usually assayed ("aggregation-competent cells," about 8–12 h of development for bacterially grown cells), only gp80 and p95 are detected by the vegetative-cell-absorbed anti-gp80 antibodies. Of these molecules, gp80 seems to be quantitatively the more prominent (by perhaps tenfold); furthermore, gp80 is highly enriched in membranes while p95 is depleted or absent. Thus, gp80 is most likely the functionally important target for the antibodies, although a role for p95 cannot rigorously be excluded. It is even conceivable that a crossreacting molecule that is not detectable by SDS-PAGE (such as a glycolipid or large carbohydrate) might be the functional target. We are now trying to approach these questions by analyzing the biochemical relationships between p95 and gp80 and by continuing our attempts to obtain an antibody preparation that reacts only with gp80. Such a reagent should make it possible to approach the question of the role of gp80, in molecular terms, in the adhesion of these cells.

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