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# Cytoskeletal Association of an Esterase in *Dictyostelium discoideum*

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## Abstract

A 70-kDa glycoprotein, gp70, was found enriched in the detergent-insoluble cytoskeletal fraction of axenically grown *Dictyostelium discoideum* cells. Its N-terminal amino acid sequence identified it as “crystal protein” (L. Bomblies *et al.*, 1990, *J. Cell Biol.* **110**, 669–679). This finding was corroborated when antibody to crystal protein cross-reacted with gp70 and its deglycosylated form. The postulated esterase activity of gp70/crystal protein was verified through comparative enzyme assays of extracts derived from cells that either overexpressed or lacked gp70. Gp70 cosedimented with cytoskeletons on sucrose gradients, suggesting an interaction with the cytoskeleton. Coisolation of gp70 with detergent-extracted cells, observed by immunofluorescence microscopy, also implied a gp70-cytoskeletal association. These data supported the idea that the localization or secretion of gp70, or both, was cytoskeletally mediated. Although axenically grown cells contained high levels of gp70, the same cell lines had reduced levels of gp70 when grown in bacterial suspension or in nutrient media containing bacteria. Bacterially grown cells, compared to axenically grown cells, had lower fluid-phase uptake rates even when nutrient media was present, indicating that phagocytosis was a preferred mode of feeding. Thus, bacteria inhibited gp70 expression, which suggested a role for prestarvation factor, in regulating its synthesis.

**Keywords:** *Dictyostelium discoideum*, glycoprotein, esterase, cytoskeleton, pinocytosis, phagocytosis

**Abbreviations:** AP, alkaline phosphatase; Con A, concanavalin A; FITC-dextran, fluorescein isothiocyanate-dextran; gp, glycoprotein; mAb, monoclonal antibody; PSF, prestarvation factor

## INTRODUCTION

The two forms of endocytosis, pinocytosis and phagocytosis, differ in that the actin-based cytoskeleton is involved in the ingestion phase of phagocytosis but is not considered essential to the ingestion of solutes and fluids [1]. We are seeking a better understanding of the physiological differences between phagocytically active cells and cells engaged

in pinocytosis, especially modifications of interactions between the cytoskeleton and the cell membrane. Our premise was that membrane glycoproteins that remain associated with the detergent-insoluble (cytoskeletal) fraction [2, 3] would be potentially useful for analyses of membrane protein interactions with the actin-based cytoskeleton. Con A<sup>2</sup>-binding was used initially to detect membrane glycoproteins, and we have been able to discern differences in cytoskeletally associated glycoproteins between axenically and bacterially grown cells, one of which has been suggested to be involved in phagocytosis [4]. In this report, we describe a 70-kDa glycoprotein, gp70, that was detected in cytoskeletons prepared from axenically grown cells. Our studies indicated that gp70 was neither a membrane protein nor involved directly in the phagocytosis process. We found, however, that gp70 was associated consistently with the cytoskeleton and that its expression in vegetative cells was suppressed by either bacteria or the process of phagocytosis.

N-terminal amino acid sequence obtained from gel-purified gp70, extracted initially by Concanavalin A chromatography of cytoskeletons, identified gp70 to be the same molecule as a “crystal protein” previously characterized by Bomblies *et al.* [5]. These investigators found crystal protein to be a major species in crystalline inclusion bodies of developing *Dictyostelium discoideum* cells. Because its deduced amino acid sequence revealed sequence similarity with known esterases (and lipases; [6]), the vesicles containing crystal protein and a companion protein D2 (also having esterase homology) were called esterosomes [5]. Crystal protein and D2 were suggested to be required for spore wall degradation [5].

In this report, we provide evidence that gp70/crystal protein had esterase activity and was associated with the cytoskeleton, and we also describe its expression under different culture conditions. Comparative enzyme assays using detergent-solubilized extracts from cells that either overexpressed or lacked gp70, confirmed the esterase activity of gp70. Cosed-

imentation of gp70 with detergent-insoluble cytoskeletons on sucrose gradients suggested an interaction of gp70 with the actin-based cytoskeleton. The presence of gp70 in rhodamine phalloidin-staining cytoskeletons examined by immunofluorescence microscopy also supported a connection between gp70 and the cytoskeleton. The cytoskeletal association of gp70 may have a role in either positioning the esterosomes containing gp70 and D2 during or after their synthesis, or regulating their eventual secretion for the postulated function of spore wall lysis.

Similar to other proteins in *D. discoideum* [7–10], levels of gp70 were affected by whether cells were axenically or bacterially grown. In vegetative (log-phase) cultures of axenic strains nutrient media yielded high levels of gp70 expression and bacterial suspensions reduced gp70 expression, and in wild-type cells solely bacterially grown, gp70 was absent. Stimulation of gp70 synthesis by nutrient media was one explanation for the differences in gp70 levels. However, fluid phase uptake measurements indicated that cells reduced their pinocytic activity and became phagocytically active when provided with heat-killed bacteria even after a history of growth in nutrient media or if bacteria and nutrient media were simultaneously available. Given a choice, axenic cells apparently preferred phagocytosis over pinocytosis to ingest nutrients. Thus, instead of nutrient media promoting its synthesis, it appeared that gp70 expression was inhibited by bacteria.

## MATERIALS AND METHODS

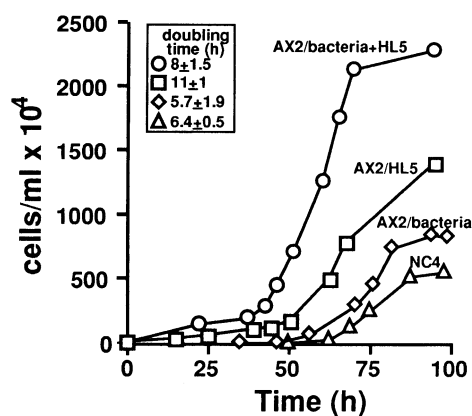
**Cell growth and cytoskeletal preparation.** *D. discoideum* cells (AX2) were grown to  $6$  to  $8 \times 10^6$  cells/ml at  $20^\circ\text{C}$  in HL5 media (nutrient media; [12]). Two transformed cell lines, one that overexpresses crystal protein (AT-K2) and one that lacks crystal protein expression (AT-K<sub>neg</sub>), were generated using standard methods [13]. These strains were grown in HL5 containing  $10 \mu\text{g/ml}$  G418. For growth of NC4 (provided by Dr. M. Clarke, Oklahoma Medical Research Foundation, Oklahoma City, OK) and the axenic strains in bacterial suspensions, *Klebsiella aerogenes* (Dr. Clarke) was grown on nutrient agar (SM) plates [14], harvested, and washed in Sorensen's phosphate buffer ( $14.6 \text{ mM KH}_2\text{PO}_4$ ,  $2 \text{ mM Na}_2\text{HPO}_4$ , pH 6.1) and then heat-killed by autoclaving for 20 min. This provided a uniform stock preparation of bacteria that also would not grow when combined with nutrient media. Bacteria were suspended to an OD600 of 10 in either sterile Sorensen's buffer or HL5 when conducting cell growth experiments.

Cell growth was monitored, by counting cells using a haemocytometer, for the following culture conditions: AX2 cells grown in HL5, heat-killed bacteria or heat-killed bacteria resuspended in HL5, and NC4 cells grown on heat-killed bacteria (Figure 1). From the average of three growth curves, doubling times of the cells were calculated (Figure 1, inset). The cultures grown in suspensions solely of bacteria had shorter generation times than cells grown in HL5 or HL5 plus bacteria, although not as short as the 3 to 4 h reported for some strains [15]. Also, the bacterially grown cells reached stationary phase at densities of  $5$  to  $8 \times 10^6$  cells/ml, which were lower than the densities typically reached by cells provided nutrient media. A possible explanation for the different growth characteristics was that the autoclaved bacteria were not as readily digested as untreated bac-

teria. To minimize carryover of gp70 when axenically grown AX2 were switched to bacteria or liquid media plus bacteria, cells were transferred at low density ( $1$  to  $2 \times 10^5$  cells/ml), grown, and passaged for a minimum of 48 h before their use in assays. For analyses of secreted protein, media from high-density cultures ( $8$  to  $9 \times 10^6$  cells/ml of cells grown in liquid media;  $4$  to  $5 \times 10^6$  cells/ml of cells grown in bacterial suspension) first was centrifuged at  $27,000g$  for 30 min to remove cell debris and then at  $100,000g$  for 1.5 h before concentrating fivefold with a Centriprep 30 (Amicon Corp., Beverly, MA).

Cytoskeletons were prepared from cells in mid-log growth [16] in the presence of  $5 \text{ mM}$  benzamidine hydrochloride and  $1 \text{ mM}$  phenylmethylsulfonyl fluoride. Cell extracts were prepared by forced lysis through  $5\text{-}\mu\text{m}$  Nucleopore filters (Corning Costar, Corning, NY) following a previously described procedure [17]. Sucrose solutions for step gradients had the same composition as the buffer used for preparing samples. Step gradients ( $4.2 \text{ ml}$  total volume) were generated with  $600 \mu\text{l}$  of  $30\%$  (w/v) and  $800 \mu\text{l}$  each of  $40$ ,  $50$ ,  $60$ , and  $80\%$  (w/v) sucrose solutions. Cytoskeletal preparations or cell extracts ( $400 \mu\text{l}$  of  $5 \times 10^7$  cells/ml) were loaded on gradients that were centrifuged at  $55,000 \text{ rpm}$  in an SW60 rotor (Beckman Instruments, Palo Alto, CA) for 1 h at  $4^\circ\text{C}$ . After puncturing the bottom of tubes,  $200\text{-}\mu\text{l}$  fractions were collected and denatured immediately for electrophoresis on  $10\text{--}20\%$  acrylamide gradient gels. Sucrose gradient experiments were repeated a minimum of three times for each preparation.

**Pinocytosis assay.** The pinocytic activity of AX2 cells grown under the three nutrient conditions (HL5, heat-killed bacteria, or heat-killed bacteria resuspended in HL5) and of NC4 cells grown on heat-killed bacteria was compared. Fluorescein isothiocyanate (FITC)-dextran (Sigma Chemical Co., St. Louis, MO) was added to shaken, log-phase cell cultures to a final concentration of  $2 \text{ mg/ml}$  [18]. Aliquots of  $1$  or  $2 \text{ ml}$  were removed at the times indicated and diluted into  $14 \text{ ml}$  of ice-cold  $50 \text{ mM}$  sodium phosphate buffer, pH 9.2, containing  $0.2\%$  sodium azide. Cells then were collected by a  $10 \text{ min}$  centrifugation at  $400g$ . The supernatant was aspirated and the cells were washed at least three times with the same buffer. Equal numbers of cells were then lysed in  $50 \text{ mM}$  sodium phosphate buffer, pH 9.2, containing  $0.2\%$  SDS and heated at  $80^\circ\text{C}$  for  $10 \text{ min}$ . After cooling to room temperature and clarification by centrifugation,



**Figure 1.** Cell growth and doubling time in suspension culture. AX2 cells were grown in HL5, nutrient media (squares), with heat-killed bacteria (diamonds), or with heat-killed bacteria in HL5 (circles). NC4 cells were grown with heat-killed bacteria (triangles). Representative curves for each condition are presented and offset along the time (x)-axis for clarity. Doubling times (inset) are the mean of three experiments  $\pm$  SEM.

the fluorescence of the samples was measured in a Perkin-Elmer LS-5B Luminescence Spectrometer (Norwalk, CT) using 470 nm and 520 nm for excitation and emission wavelengths, respectively.

**Protein gels and blots.** Protein was assayed by the Lowry method using bovine serum albumin as a standard [19]. Sodium dodecyl sulfate (SDS) polyacrylamide gels [20] were either silver [21] or Coomassie blue stained [22]. Relative molecular masses were determined using prestained molecular weight standards (Life Technologies, Gaithersburg, MD) of myosin heavy chain (216 kDa), phosphorylase  $\beta$  (110 kDa), bovine serum albumin (71 kDa), ovalbumin (43 kDa), carbonic anhydrase (28 kDa),  $\beta$ -lactoglobulin (18 kDa), and lysozyme (15.4 kDa). Electrophoretically separated proteins were transferred to nitrocellulose (0.45  $\mu$ m pore size; Schleicher & Schuell, Keene, NH) using a tank blotter [23].

Protein blots were probed either with crystal protein (mouse) antibody 129-202-6 ([5]; see below), anti-actin (rabbit) antibody (Sigma), or biotinylated Con A (Sigma) followed by the appropriate (anti-mouse, anti-rabbit or streptavidin) alkaline phosphatase (AP) conjugate (Sigma) using previously described procedures [4, 24]. Hybridomas producing monoclonal antibodies 130-80-2 (mAb 80) and 129-202-6 (mAb 202) specific for crystal protein [5] (called gp70 in this study) were provided by Dr. G. Gerisch (Max-Planck-Institut für Biochemie, Germany) and grown in media containing 10% fetal calf serum, 2 mM glutamine, 0.1 mg/ml kanamycin, and 0.01 mM  $\beta$ -mercaptoethanol in RPMI 1640 (Life Technologies).

**Isolation, gel purification, and N-terminal sequencing of crystal protein (gp70).** Cytoskeletons, prepared as described above, were solubilized overnight at 4°C in 20 mM 2-[N-morpholino]ethane sulfonic acid, pH 6.0, 500 mM NaCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , and 2.5% Triton X-100 (MES buffer). Calcium aided in disrupting the actin microfilaments, presumably through calcium-dependent filament severing proteins and calcium-inactivated filament cross-linking proteins [25]. The solubilized sample was diluted with an equal volume of cold water and incubated overnight at 4°C with Con A agarose beads (Sigma). After washing with ten bed volumes of half-strength MES buffer, the Con A beads were incubated overnight at 4°C with two bed volumes of 500 mM methyl  $\alpha$ -D-mannopyranoside in half-strength MES buffer. The eluted sample was warmed to 70°C for 7 min to cause a phase separation of the Triton X-100. The aqueous phase was discarded and SDS was added to 2% of the detergent phase, which was then run on an 8% acrylamide SDS-gel. Gels were stained with a nonfixing method [26]. Bands corresponding to gp70 were excised from the gels and protein was electroeluted from the gel slices and concentrated using an Amicon Centri-Eluter following manufacturer's instructions.

Gel-purified gp70 was run on a 10% SDS-gel and blotted to Pro-Blott Membrane (Applied Biosystems, Foster City, CA). Blotted protein was detected by Amido Black staining as recommended by Applied Biosystems. The visualized protein band was cut out, rinsed with deionized water, air-dried and sent to the Protein Structure Core Facility (University of Nebraska Medical Center, Omaha) for N-terminal sequencing.

**Deglycosylation.** Recombinant peptide-N-glycosidase F (PNGase F; Boehringer Mannheim, Indianapolis, IN) was used to cleave N-linked oligosaccharides from gel-purified gp70, following vendor guidelines. Electroeluted gp70 (approximately 5  $\mu$ g) was heated for 7 min at 80°C in 20 mM sodium phosphate, pH 7.2, 25 mM EDTA, 1% (v/v) 2-mercaptoethanol, 0.1% SDS. The sample was cooled, and Triton X-100 was added to 1% of the total volume. PNGase F (1 U) was added, and the sample was incubated at 37°C for 24 h. The reaction was stopped by adding SDS to a final concentration of 0.7%.

**Esterase assays.** Cytoskeletons, prepared as described, were isolated from equal numbers of AX2, AT-K2, and AT-K<sub>neg</sub>, all grown

axenically. The cytoskeletons were solubilized with Triton X-100 and incubated with Con A agarose beads, as described above. The protein eluted with methyl  $\alpha$ -D-mannopyranoside was assayed for total protein and tested for esterase activity using a modified method of Gu and Zera [27, 28].

Esterase assays were performed in 96-well microtiter plates read at 605 nm with a Bio-Tek Instruments Autoreader EL311 (Winsok, VT). Sample or buffer (1 to 20  $\mu$ l) was added to 185  $\mu$ l of 50 mM 3-(4-morpholino)propanesulfonic acid buffer containing 0.25 mM naphthol acetate (initially dissolved in ethanol). With respect to equal cell numbers, extracts from AT-K2 cells reached maximum absorbances in less time than the extracts from AT-K<sub>neg</sub>. The assays were incubated at 30°C for 10 to 30 min depending on the extract being used. The reactions were stopped with 30  $\mu$ l of 0.3% Fast Blue Salt BN (Sigma), 3.5% SDS. Absorbances were converted to concentrations using a standard curve generated from known amounts of  $\alpha$ -naphthol. A series of control experiments were performed to establish assay conditions that resulted in the linear production of  $\alpha$ -naphthol. Both concentrations and incubation times were varied to determine optimal assay conditions of each of the gp70-enriched extracts of the three cell lines. By determining where each sample fell in the linear portion of a naphthol standard curve, incubation times and volumes for each sample were determined. Esterase activities of extracts from each cell line were normalized to protein content. Differences in assay times were factored into the calculations of total naphthol turned over per min per mg protein.

**Microscopy.** Cells and Cytoskeletons [29] were fixed and permeabilized using the agar overlay method [30]. To detect gp70 (crystal protein), hybridoma cell culture supernatant containing mouse mAb 80 was used [5], followed by FITC-anti-mouse IgG (Pierce Chemical Co., Rockford, IL) adsorbed against glutaraldehyde-fixed AX2 [31]. Rhodamine phalloidin was used (Molecular Probes, Eugene, OR) to stain filamentous (F-) actin. Samples were examined with a Nikon Diaphot equipped with epifluorescence optics to visualize rhodamine and fluorescein signals and a 60 $\times$  Plan Apochromat oil objective (NA 1.4). Photographs were taken with Kodak T-Max 400 film developed with D-76 (Eastman Kodak, Rochester, NY). Images on negatives were scanned with a Polaroid Sprint Scan and imported into Adobe Photoshop for Macintosh.

## RESULTS

### Gp70 in Axenically Grown Cells

Detergent-insoluble (cytoskeletal) fractions of axenically grown *D. discoideum* amoebae displayed a strong Con A-binding signal at 70 kDa and weaker signals at 105, 32, and 27 kDa (Figure 2, lane 1). Cytoskeletons of bacterially grown cells had a prominent Con A-binding species at 18 kDa and weaker Con A-staining signals at 130, 105, 32, and 27 kDa (Figure 2, lane 2). The Con A-binding signals were absent when blots were probed with biotin Con A in the presence of 200 mM methyl  $\alpha$ -D-mannopyranoside (data not shown). A signal not abolished by the competing sugar was observed at 72 kDa (species indicated with open arrow) in both preparations. We investigated further the strong Con A-binding signal at 70 kDa, which we called gp70, in Cytoskeletons from cells grown in nutrient media (species in lane 1 indicated with a solid arrow, Figure 2).

Gp70 from Cytoskeletons was isolated by Con A chromatography and gel-purified (see Materials and Methods). An N-

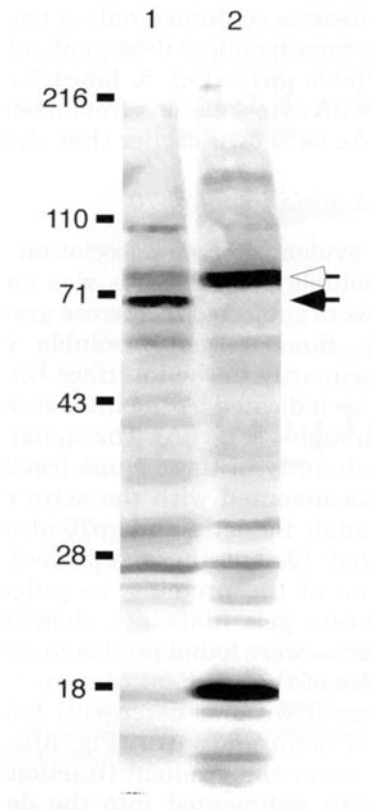
terminal sequence of 20 residues from gel-purified gp70 was obtained (RKGIR TLGDN EVLLS DGAIR) and a database search showed it to be an exact match to residues 25 to 44 of the previously described “crystal protein” [5]. The N-terminus of gp70 was five residues shorter than the biochemically determined N-terminus of crystal protein [5]. This shorter sequence was possibly a consequence of proteolysis during the purification procedure.

Crystal protein was suggested to be a glycoprotein based on the difference in its mobility on SDS-gels ( $M_r$  69 kDa) and its size, 59 kDa, based on the deduced amino acid sequence [5]. The binding of Con A by gp70 was consistent with the predicted N-linked glycosylation of crystal protein [5] and confirmed by the enzymatic deglycosylation of gel-purified gp70 (Figure 3). PNGase-treated (gel-purified) gp70 shifted in size to 64 kDa (Figure 3A) and no longer bound Con A (Figure 3B). The deglycosylated form of gp70 had an apparent molecular mass on SDS-gels approximately 5 kDa larger than the calculated mass of 59 kDa for crystal protein [5]. Because omission of reductant did not alter the mobility of untreated gp70, the apparent mass difference apparently was not due to intact disulfide bonds (data not shown). Gp70 and its deglycosylated form were confirmed to be crystal protein by

their reactivity with mAb 202 (Figure 3C). A slight amount of proteolysis of gel-purified gp70 was detected by both Con A (Figure 3B) and mAb 202 (Figure 3C).

Esterase Activity of gp70

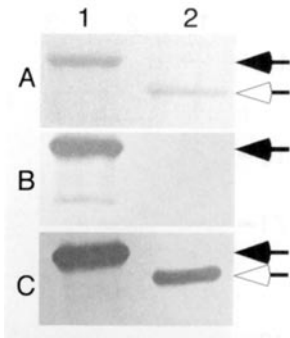
Crystal protein, with roughly 30% sequence homology to known esterases, was suggested to have esterase activity [5]. Biochemical assays testing its enzymatic ability, however, were not presented. The availability of cells that overexpressed crystal protein (AT-K2) or lacked crystal protein (AT-K<sub>neg</sub>) [13] called gp70 in this report, allowed comparative analyses of esterase activities. Extracts enriched in gp70, through Con A chromatography of solubilized cytoskeletons, from AX2, AT-K2, and AT-K<sub>neg</sub> had esterase activities that corresponded to their relative content of gp70. There was a 6.5-fold difference in esterase activity between AX2 and AT-K2 and a close to 50-fold difference between AX2 and AT-K<sub>neg</sub> (Table 1). Gp70 was the species in the protein profiles that differed significantly in quantity (Figure 4). Both the silver-stained gel of the extracts (Figure 4A) and blotted samples (Figure 4B) confirmed that AT-K2 had high levels of gp70 (lane 3) compared to AX2 (lane 2), and AT-K<sub>neg</sub> (lane 1) lacked gp70.



**Figure 2.** Con A-binding proteins were found in detergent-insoluble cytoskeletons. Protein blot, of a 10–20% acrylamide gradient SDS-gel loaded (40  $\mu$ g protein/lane) with cytoskeletons from axenically grown (lane 1) or bacterially grown (lane 2) cells, probed with biotin-labeled Con A followed by streptavidin AP. Masses of molecular weight markers (in kDa) are indicated to the left of lane 1. The solid arrow points to a 70-kDa species in lane 1, and the open arrow indicates a false doublet signal that was observed in the absence of biotin-Con A.

**Table 1.**

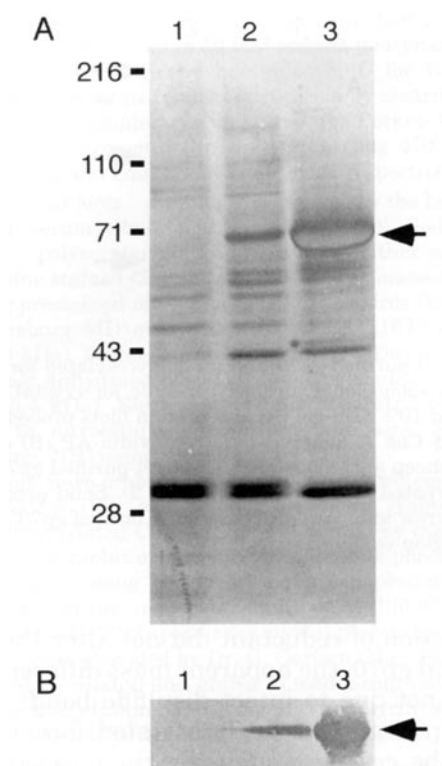
Esterase Activities of Fractions from Different Cell Lines Enriched in gp70 by Con A Chromatography	
Strain	Activity (means $\pm$ SEM) $\mu$ mol/min/ $\mu$ g protein
AX2	0.0248 $\pm$ 0.003
AT-K2	0.1602 $\pm$ 0.017
AT-K <sub>neg</sub>	0.0005 $\pm$ 0.002



**Figure 3.** Gel-purified gp70 and its deglycosylated form were recognized by a monoclonal antibody specific for crystal protein [5]. Silver-stained 10% SDS-gel (A) and protein blots probed with either biotin-labeled Con A followed by streptavidin-AP (B) or mAb 202 followed by sheep anti-mouse-AP (C) of gel-purified gp70 untreated (lane 1) or treated with PNGase F (lane 2). Solid arrow indicates gp70; open arrow indicates deglycosylated form of gp70. Only the 60 to 75 kDa region is shown.

Overall, there were substantial differences in mean esterase activities between cell lines. Using Bonferroni's method for multiple comparison [32], a one-way ANOVA was computed using 95% confidence intervals. Esterase means differed between AX2 and AT-K2 ( $P < 0.01$ ) and between AX2 and AT-K<sub>neg</sub> ( $P < 0.002$ ). Combined with the observed expression levels of gp70 (Figure 4), these statistically significant differences support the hypothesis that gp70 had esterase activity.

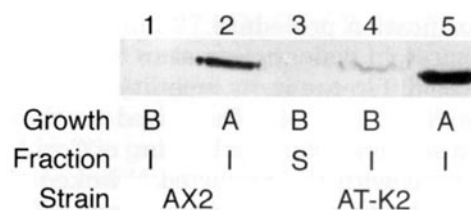
With the antibody specific for gp70, we corroborated the initial observation that a 70-kDa glycoprotein (Figure 2), identified subsequently as gp70, was present in detergent-insoluble cytoskeletons of axenically grown AX2 cells but absent from cytoskeletons of vegetative AX2 cells grown on bacteria to low densities (Figure 5, compare lanes 1 and 2). Similarly, detergent-insoluble cytoskeletons of bacterially grown AT-K2 cells of low cell density cultures contained only a trace amount of gp70, and the corresponding detergent-soluble fraction had no detectable gp70 (Figure 5, lanes 3 and 4). Gp70 fractionated with cytoskeletons from axenically grown AT-K2 (lane 5), as shown earlier (Figure 4, lane 3).



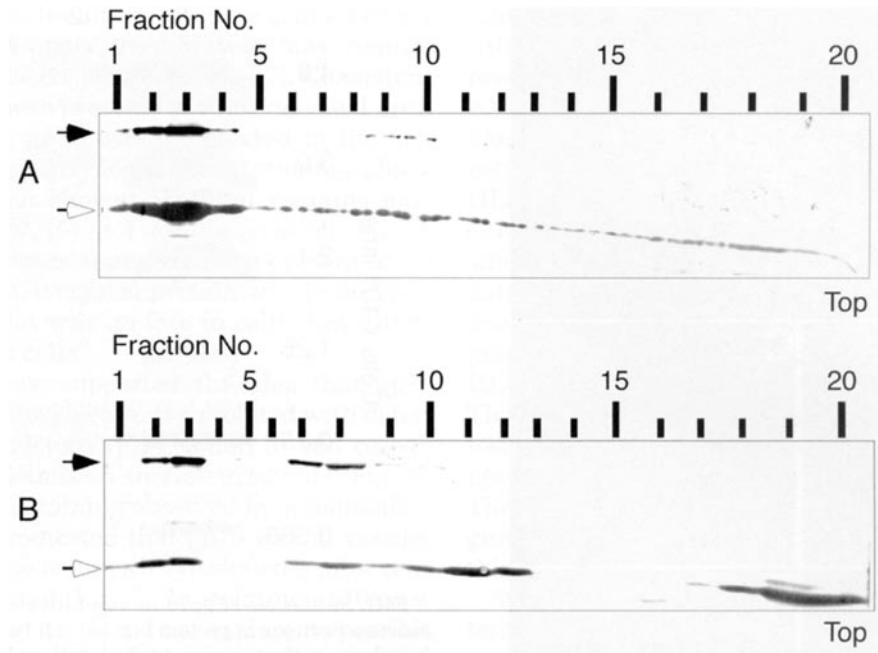
**Figure 4.** Gp70 and other glycoproteins were enriched by Con A chromatography of solubilized cytoskeletons derived from axenically grown cells. Methyl  $\alpha$ -D-mannopyranoside eluates of AT-K<sub>neg</sub> (lane 1), AX2 (lane 2), and AT-K2 (lane 3) were run on a silver-stained 10% SDS-gel (A) and also blotted to nitrocellulose (B), which was probed with mAb 202 followed by sheep anti-mouse-AP. Each lane contains the equivalent of  $5 \times 10^5$  cells. Arrows indicate gp70. Molecular weight markers (in kDa) are indicated to the left of Panel A.

### Cytoskeletal Association of gp70

Additional evidence for an association of gp70 with detergent-insoluble cytoskeletons was obtained when cell extracts were subjected to sucrose gradient centrifugation (Figure 6). Detergent-insoluble cytoskeletons sedimented primarily to the interface between 60 and 80% sucrose, as indicated by the intense actin signal in fractions 2 through 4 (Figure 6A). The signal for gp70 was found predominantly in these same fractions, indicating it had cosedimented with the actin-rich cytoskeletons. The signals for actin and gp70 also coincided in fractions 9 and 10, but these represented relatively small amounts of the proteins, as judged by silver-stained duplicate gels (data not shown). Other Con A-binding species were found predominantly in the less dense fractions of the gradient (data not shown). Cell lysates, prepared without detergent, had a different distribution of actin and gp70 (Figure 6B). Most of the actin did not enter the gradient (fractions 18, 19, and 20). Some actin sedimented into the denser sucrose (fractions 2 and 3) where the most intense gp70 signal also was seen. The other strong actin signal observed midway through the gradient (fractions 11 and 12) did not coincide with the second strongest signal for gp70 (fractions 7 and 8). Because crystal protein was postulated to be a secreted protein [5], we suggest that the two sedimentation locations of gp70 in fractionated cell lysates represented two populations of vesicles. One population retained an association with actin-rich cytoskeletons (fractions 2 and 3) that remained stable during cell lysis, and was the dominant population when cytoskeletons were intentionally prepared (Figure 6A). The other population (fractions 7 and 8) represented vesicles that lost their interaction with the cytoskeleton either because the conditions for lysis disrupted the interaction or the cytoskeleton itself.



**Figure 5.** Gp70 levels were reduced in cells grown in bacterial suspension. Section of protein blot of detergent-insoluble cytoskeletons (I; lanes 1, 2, 4, 5) or the detergent-soluble fraction (S; lane 3) prepared from mid-log phase cells either bacterially (B; lanes 1, 3, 4) or axenically grown (A; lanes 2, 5) probed with mAb 202 followed by sheep anti-mouse AP. Lanes 1–2: AX2; lanes 3–5: AT-K2. Each lane was loaded with the equivalent of  $1 \times 10^6$  cells.



**Figure 6.** Gp70 cosediments with actin-rich cytoskeletons. Blots of fractions from sucrose step gradients loaded with cytoskeletons (A) or cell lysates (B) were probed with mAb 202 reactive with gp70 (solid arrow) and anti-actin (open arrow) followed by the specific AP-secondary antibodies. The high concentration of sucrose in the lower portion of the gradient retarded the migration of proteins on SDS-gels. Fraction 20 is from the top of the gradient.

The cytoskeletal association of gp70 was confirmed additionally through immunofluorescence microscopy (Figure 7). In axenic AX2 cultures, permeabilized cells had discrete spots distributed throughout the cell (A) that presumably corresponded to the “esterosomes” containing crystal protein described by Bomblies, *et al.* [5]. Detergent (Triton X-100)-extracted cells exhibited a similar punctate pattern when stained with mAb 80 (B) and were enriched in insoluble, cytoskeletal (filamentous) actin, as seen by their rhodamine-phalloidin staining (C). The FITC-goat anti-mouse secondary antibody alone stained neither cytoskeletons (D) nor permeabilized cells (E).

#### *Gp70 Expression Levels and Growth Conditions*

The levels of gp70 in cells varied depending on their nutrient source. Lysates from mid-log phase cells were compared on immunoblots (Figure 8). Using the cell number at which the gp70 signal disappeared ( $0.075 \times 10^6$  cells/lane; data not shown), axenically grown AX2 cells (lane 1), had approximately 10- and 15-fold higher levels of gp70 than cells grown in nutrient media containing bacteria (bacteria/HL5; lane 2) and bacterially grown cells (lane 3), respectively. Cells grown in bacteria/HL5 (lane 2) had 1.3-fold more gp70 than cells grown in bacteria alone (lane 3). Gp70 was absent from NC4 cells (lane 4), which are exclusively phagocytic. Gp70 expression thus appeared to be en-

hanced in pinocytic cells compared to phagocytic cells. NC4 from late-log and stationary cultures expressed gp70 (data not shown). This was expected, given the finding that gp70 is developmentally regulated [5].

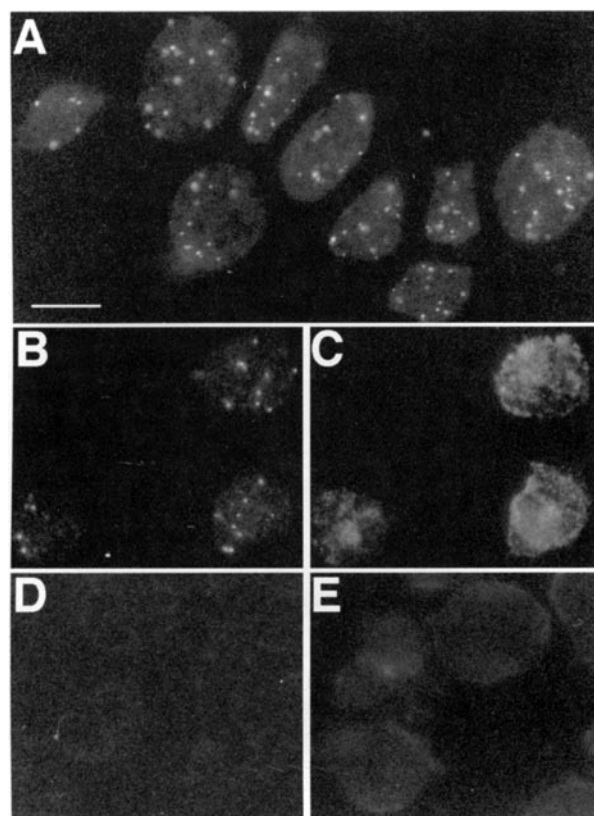
Fluid-phase uptake studies verified that there were differences in the apparent pinocytic abilities of mid-log phase cultures grown with different food sources (Figure 9). High fluid-phase uptake rates were exhibited by axenically grown AX2 cells (squares). Cells provided only with heat-killed bacteria had relatively low fluid-phase uptake rates (AX2, diamonds; NC4, triangles). AX2 cells, given a choice between pinocytosis and phagocytosis, appeared to favor primarily phagocytosis (circles). Their fluid-phase uptake, although modest, was consistently greater than that of cells fed solely bacteria but still significantly lower than that of their counterparts in nutrient media. These studies lent support to the idea that gp70 expression was inversely correlated with phagocytic growth.

## DISCUSSION

We have shown that a 70-kDa cytoskeletally associated glycoprotein, gp70, was the same as the crystal protein previously described by Bomblies *et al.* [5]. The binding of Con A by gp70 (Figure 2) was predicted by protein sequence [5]. Gp70 lost its ability to bind Con A when treated with PNGase

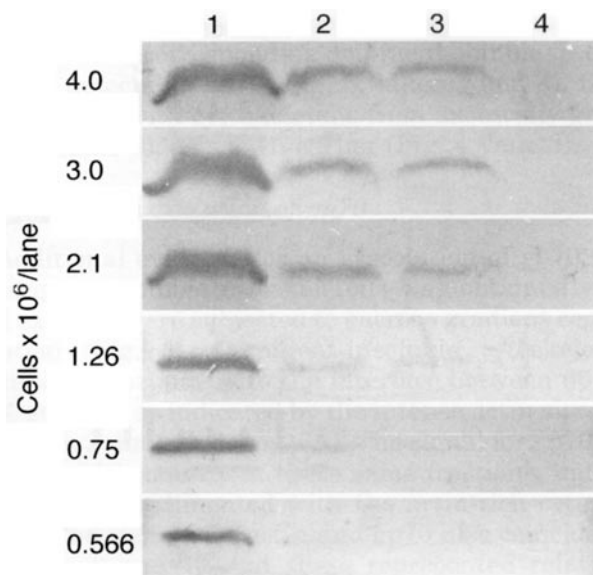
F (Figure 3), an enzyme that recognizes N-linked oligosaccharides [33], indicating that the carbohydrate chain(s) were attached to asparagine residues. The identification of gp70 as crystal protein was made initially by matching the N-terminal 20 amino acids of gel-purified gp70 to crystal protein sequence. This finding was confirmed when both gp70 and its deglycosylated species were recognized by mAb 202, which is specific for crystal protein [5] (Figure 3). The deglycosylated form of gp70 still had an apparent molecular mass on gels approximately 5 kDa larger (Figure 3) than the calculated mass (59 kDa) for crystal protein [5]. This difference may be due to undetected modifications to the protein or features of the protein that retard its mobility in SDS-PAGE.

Our assays for esterase activity support the suggestion that gp70 was an esterase (Table 1), as predicted by its similarity in amino acid sequence to other esterases and lipases [5, 6]. Esterase activity was high in detergent-solubilized extracts enriched in Con A-binding proteins from AT-K2 cells, which overexpressed only gp70, and negligible in extracts of AT-K<sub>neg</sub> cells, which lacked solely the gene for gp70 (Table 1 and Figure 4). Both gp70/crystal protein and D2, the other protein

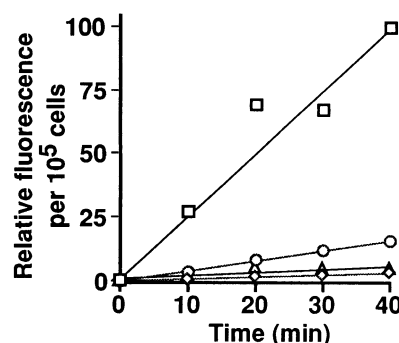


**Figure 7.** Immunofluorescence labeling of gp70 in permeabilized or detergent-extracted cells. Whole cells (A) and cytoskeletons (B and C) were stained with mAb 80 followed by FITC-goat anti-mouse IgG. Cytoskeletons also were stained with rhodamine phalloidin (C). B and C show the same field of cells. As controls, cytoskeletons (D) or cells (E) were stained with FITC goat anti-mouse IgG only. Bar = 10  $\mu$ m.

with homology to esterases, were postulated to be liberated from the esterosomes in order to be functional [5]. Madea and Takeuchi [34] and Cotter *et al.* [35] observed that crystalline bodies disappeared during spore germination, leading Bomblies *et al.* [5] to suggest that the esterase activity of gp70/crystal protein and D2 is required to break down spore coats. Others have suggested that spore germination may require the release of degradative enzymes [35–37]. Consistent with the idea that these proteins are not released until late in development, gp70 was undetected in the medium of cultures from



**Figure 8.** Levels of gp70 in vegetative cells were affected by food sources. Sections of protein blots of cell lysates (prepared from mid-log phase cultures) were probed with mAb 202 followed by sheep anti-mouse AP. AX2 cells were grown either axenically in HL5 (lane 1), in nutrient media (HL5) containing heat-killed bacteria (lane 2), or in bacterial suspension (lane 3). NC4 cells were grown in bacterial suspension (lane 4). Cell numbers loaded of each sample are indicated to the left of each panel.



**Figure 9.** Fluid phase uptake by mid-log phase cells with different sources of food. Pinocytosis was measured by monitoring FITC-dextran uptake [18] of AX2 suspension cultures growing in HL5 (squares), heat-killed bacteria (diamonds), or HL5 plus heat-killed bacteria (circles), and NC4 growing in heat-killed bacteria (triangles). Data (all points are averages of duplicate samples) are from a representative experiment.



early log to late stationary phase cultures [our data not shown; 5]. What remains puzzling is Bomblies *et al.* [5] and our observation (Figure 7) that gp70/crystal protein appeared to be present in all cells. If the role of gp70/crystal protein was to degrade spore coats, then what was its fate in cells that differentiate into prestalk cells?

Biochemical evidence supported the idea that gp70 interacted with the cytoskeleton. It coisolated with detergent-insoluble cytoskeletons (Figs. 2 and 5) and cosedimented with cytoskeletons on sucrose gradients (Figure 6). Further, the punctate staining observed by immunofluorescence microscopy indicated that gp70 was in vesicles that remained with the actin-rich cytoskeleton after cells were detergent extracted (Figure 7). In isolating gp70/crystal protein, Bomblies *et al.* [5] did not explore its possible association with detergent-insoluble cytoskeletons. They did, however, raise the questions of how gp70/crystal protein and the D2 protein are cocrystallized, sorted from the RER, and subsequently secreted. Cytoskeletal elements, most notably microtubules and associated proteins, are responsible for vesicular trafficking and delivery of proteins to the cell surface [38]. Actin microfilaments also appear to play a role in vesicle transport and positioning, as well as regulating vesicle fusion with the plasma membrane. In *D. discoideum*, the actin-binding and vesicle-associated protein comitin is suggested to link vesicles to the cytoskeleton [39,40]. The involvement of the actin cytoskeleton in exocytosis is postulated for the polarized secretion of vesicles in budding yeast [41]. At nerve synapses, the exocytosis of synaptic vesicles appears to be regulated through an association, mediated through synapsins, with the neuronal cytoskeleton, including actin microfilaments [42]. Actin microfilaments appear to facilitate the fusion of mature endosomes with preexisting lysosomes in human carcinoma (HEp-2) cells [43]. As with these examples of microfilament associations with vesicles, our findings are consistent with the idea that gp70/crystal protein along with the D2 protein [5] were in esterosomes linked to the cytoskeleton. This linkage presumably mediated esterosome biosynthesis or localization, or possibly had a role in regulating the secretion of gp70/crystal protein and D2 for spore coat degradation.

Gp70 expression was correlated to the food source of the *D. discoideum* cells. Expression levels of some proteinases isoforms [10] and lysosomal enzymes [9] in *D. discoideum* are affected by whether cells acquire nutrients through phagocytosis or pinocytosis. Bacterially grown AX2 and AX2 cells grown in nutrient media containing heat-killed bacteria had 15-fold and 10-fold less gp70, respectively, than axenically grown AX2 cells (Figure 8). Pinocytosis assays revealed that when both HL5 and bacteria were present, AX2 *D. discoideum* had fluid-phase uptake rates more like NC4, which are strictly phagocytic (Figure 9). AX2 cells behaved primarily as phagocytes even in the presence of HL5, and thus did

not internalize as much HL5 as axenically grown AX2. Also, gp70 levels of AT-K2 were similar to that of AX2 when grown in bacteria (Figure 5 and data not shown), implicating a response to bacteria by the axenic cells. From these data, we suggest that bacteria or phagocytosis, or both, restricted gp70 expression and that HL5 did not have an enhancing effect on gp70 expression. The pinocytosis data agreed with the idea that phagocytosis, which presumably demands more energy than pinocytosis [1], was preferred by *D. discoideum* amoebae. The faster doubling times, and therefore more efficient growth, of bacterially grown cells (Figure 1) also supported this observation.

We speculate that the apparent suppressive effect of bacteria on gp70 expression in axenic strains and the negligible expression of gp70 by vegetative NC4 cells may be related to the different starvation responses by axenic and wild type *D. discoideum* cells. During vegetative growth, cells continuously secrete PSF, an autocrine factor that accumulates in proportion to cell density [44]. At high PSF to bacteria ratios, which occur during late exponential growth, PSF induces expression of several products needed for cell aggregation [45]. The gene for gp70/crystal protein may be one such gene. The ability of cells to respond to PSF is inhibited by bacteria which interfere with the ability of cells to detect or respond to PSF [44]. Studies by Burdine and Clarke [46] showed that axenic cells produce higher levels of PSF than wild-type cells during exponential growth on bacteria. We hypothesize that AX2 (axenic) cells in bacterial suspension or in nutrient media containing heat-killed bacteria secreted PSF, which induced premature synthesis of gp70/crystal protein. This expression was countered by bacteria, which dampened the response of the cells to PSF.

Our notion that PSF had a regulatory role in gp70 expression is consistent with two observations. One is the relatively high levels of gp70 expressed in axenically grown strains, which could be a consequence of PSF secretion. A second observation was the apparently density-dependent expression of gp70 by bacterially-grown AX2 and NC4 (data not shown), an expected behavior of a PSF-regulated protein. The cause and effect relationship between PSF and gp70 could be tested directly by using partially purified PSF [44].

In summary, we demonstrated that a 70-kDa glycoprotein, gp70, enriched in detergent-insoluble cytoskeletons of axenically grown cells was the same molecule as crystal protein [5]. Gp70 had esterase activity, and when in esterosomes presumably with the related protein D2, had an association with the actin-based cytoskeleton that may be responsible for localizing its synthesis or regulating its secretion during spore wall degradation. The expression of gp70 was influenced by whether cells were grown in nutrient media or in bacterial suspension, which leads to the suggestion that PSF may have a role in regulating the developmental expression of gp70. Gp70 was not directly involved in the phagocytosis

sis process. Instead, its expression levels appeared to reflect the pinocytotic or phagocytotic mode of the cells. Future studies will be directed towards examining the opposing effects of bacteria and PSF on gp70 levels, as well as an investigation of the molecular basis of the gp70-cytoskeletal association.

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