GUANINE NUCLEOTIDE BINDING PROPERTIES AND ATTEMPTED IMMUNOPURIFICATION OF RAS PROTEIN FROM DICTYOSTELIUM DISCOIDEUM

By

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ABSTRACT

One purpose of this study was to determine whether the ras protein from Dictyostelium discoideum (p23) binds guanine nucleotides like the ras proteins from mammals (p21) and yeast. The other purpose of this investigation was to purify or enrich for $p23^{ras}$ from D. discoideum by immunoaffinity chromatography.

A number of different approaches were used to determine guanine nucleotide binding by $p23^{ras}$. A simple filter binding assay, binding to Western blots, and photoaffinity labeling all failed to demonstrate specific binding with lysates of D. discoideum cells. In contrast $p21^{ras}$ from transformed NIH-3T3 cell lysate was successfully photoaffinity labeled in the presence of ^{32}p-α-guanosine 5'-triphosphate (GTP) suggesting that the technique had been performed correctly. It was concluded that either $p23^{ras}$ has a very low affinity for guanine nucleotides such that GTP binding was not detectable in these experiments or that the ras protein from D. discoideum simply does not bind guanine nucleotides.

The purification of $p23^{ras}$ from D. discoideum cells was attempted in order to provide a purified protein preparation for guanine nucleotide binding and for reconstitution studies. An anti-ras monoclonal antibody (Y13-259) was used as the ligand for the immunoaffinity chromatography. This approach was not successful in that the ras protein could not be enriched relative to other proteins because the immunoaffinity columns did not bind $p23^{ras}$.

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INTRODUCTION

Dictyostelium discoideum is a cellular slime mold that provides a simple model system for the study of development (Loomis, 1982). It is unique among eukaryotes because the processes of proliferation and differentiation are separated. The vegetative amoebae proliferate in the presence of a nutrient source and upon starvation, growth stops and differentiation then occurs over a 24 hour period in the laboratory to produce two cell types. Cell-free extracts of *D. discoideum* contain a protein of molecular weight (M_r) 23,000 ($p23^{ras}$) (Pawson et al., 1985) that is specifically immunoprecipitated by an antibody originally raised against the transforming protein ($p21^{ras}$) encoded by the viral *ras* gene of Harvey murine sarcoma virus (Furth, 1982). The *ras* protein is developmentally regulated in *D. discoideum*, providing an opportunity to study the normal function of the *ras* protein during cell growth and differentiation in an organism considerably less complex than mammals.

The human *ras* oncogene family consists of three members: Harvey, Kirsten, and N-*ras*. The first two were identified as homologues of the transforming *ras* genes of Harvey and Kirsten murine sarcoma viruses (Chang et al., 1982) whereas the N-*ras* gene was originally isolated from a neuroblastoma cell line (Hall et al., 1983). Since then, cellular homologues of the *ras* gene have been identified in organisms as diverse as *Dictyostelium discoideum*, *Aplysia*, *Drosophilia melanogaster*, and *Saccharomyces cerevisiae* (Levinson, 1986).

The presence of the ras gene in normal cells and its evolutionary conservation suggest that it has an important role in cellular physiology. Transformation is likely a result of the loss of control of this normal function (Bishop, 1983). The mammalian ras proto-oncogene can be activated to a transforming oncogene by amplification of the gene's expression in the cell (Pulciani et al., 1985) or by acquiring a point mutation that results in a single amino acid change (Bos et al., 1987; Forrester et al., 1987; Quaife et al., 1987) at one of five specific residues in the encoded protein (Fasano et al., 1984; Marshall, 1986).

All three mammalian ras genes encode proteins of 21,000 M_r that are closely homologous in the N-terminal region and have a variable C-terminal domain (Gibbs et al., 1985). Although little is known as to the physiological function of the mammalian ras proteins, they bind guanine nucleotides specifically (Scolnick et al., 1979; Shih et al., 1980; Manne et al., 1984; Trahey et al., 1987) and possess a GTPase activity (Marshall, 1986). $P21^{ras}$ is located at the cytoplasmic face of the plasma membrane (Papageorge et al., 1982), anchored by a fatty acid covalently linked to the C-terminus of the protein (Sefton et al., 1982). The ability to bind GTP, the GTPase activity, and the membrane localization are all characteristics that are very similar to those of a class of regulatory G proteins that function in signal transduction across the plasma membrane (Newbold, 1984). When GTP is bound, the protein is in an active state and has an effect on a target enzyme. The hydrolysis

of GTP to GDP is thought to eliminate this signal. Transforming ras genes have a reduced GTPase activity compared to proto-oncogenes (McGrath et al., 1984; Gibbs et al., 1984) which result in an incorrectly regulated signal. Also, membrane localization of $p21^{ras}$ is required for transformation (Willumsen & Christensen, 1984) making this G protein analogy an attractive hypothesis.

Yeast RAS1 and RAS2 genes encode proteins that also possess the biochemical characteristics of G proteins (Tatchell, 1986). The RAS proteins are fatty acylated and membrane associated (Fujiyama & Tamanoi, 1986) but these properties are not essential for RAS function (Deschenes & Broach, 1987) in Saccharomyces cerevisiae. Activated RAS1 (Temeles et al., 1985) and RAS2 (Tamanoi et al., 1985) genes, with mutations analogous to those of oncogenic mammalian ras genes, encode proteins with reduced GTPase activity as compared to wild-type gene products. The conserved biochemical properties between ras proteins from yeast and mammals suggest that these characteristics may be essential to the ras gene product's cellular function. In yeast, membrane reconstitution experiments have shown that the RAS protein is involved in the activation of adenylate cyclase (Broek et al., 1985). However, purified $p21^{ras}$ does not stimulate or inhibit the adenylate cyclase of mammalian membranes (Beckner et al., 1985).

Recently, mammalian $p21^{ras}$ has been implicated in the phosphoinositide pathway of intracellular signal transduction. Inositol lipids are involved in signal transduction from

certain growth factor receptors on mammalian cell membranes to intracellular second messengers, initiating responses leading to cell growth. In response to growth facter occupation of its specific receptor, there is an activation of a phospholipase C that is specific for the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to 1,2-diacylglycerol and inositol 1,4,5-triphosphate ($1,4,5\text{-}IP_3$). These products stimulate protein kinase C and mobilize intracellular calcium, respectively (Michell & Kirk, 1986; Majerus et al., 1985). The PIP_2 is generated by sequential phosphorylation of phosphatidylinositol, by two different kinases (Berridge, 1986). There is evidence that a GTP-binding protein is involved in the regulation of phospholipase C (Cockcroft & Gomperis, 1985; Lapetina & Reep, 1987) but the protein has not as yet been characterized. Mammalian cells transformed with $p21^{ras}$ possess elevated levels of 1,2-diacylglycerol and increased protein kinase C activity compared to untransformed cells (Wolfman & Macara, 1987) and the anti-ras antibody, Y13-259, inhibits the insulin-dependent induction of Xenopus laevis oocyte maturation (Deshpande & Kung, 1987) suggesting that $p21^{ras}$ is involved in the inositol signalling pathway in response to insulin. The GDP-bound form of $p21^{ras}$ has been shown to attenuate the autophosphorylation of the insulin receptor (O'Brien et al., 1987) but its physiological significance remains unclear. Although it is tempting to postulate that $p21^{ras}$ encodes the G protein that regulates phospholipase C and interacts with the insulin receptor to

transduce a signal to this enzyme, there is no direct evidence in support of this hypothesis.

D. discoideum also has an intracellular signalling pathway involving phosphoinositide lipids although, in comparison to mammalian cells, less is known about this pathway. The signalling system of D. discoideum does not involve hormones but rather cells communicate with cyclic adenosine 5'-monophosphate (cAMP) and folate (Devreotes, 1983). Cells are attracted to folate during vegetative growth and as cells start to differentiate, they lose their sensitivity to folate and gain sensitivity to cAMP, the chemoattractant responsible for aggregation. The binding of folate or cAMP to their respective cell surface receptors induces a transient accumulation of cyclic guanosine 5'-monophosphate (cGMP), that peaks at 8 to 10 seconds after binding of the attractant (Mato et al., 1977; Wurster et al., 1977), presumably due to a transient activation of guanylate cyclase. Calcium (Small et al., 1986) and 1,4,5-IP_3 (Europe-Finner & Newell, 1985), when added to permeabilized amoebae, mimic the action of the chemoattractants and induce cGMP formation. More direct evidence for phosphoinositide involvement in the chemostatic signalling pathway was obtained when 1,4,5,-IP_3 was shown to accumulate rapidly in response to stimulation by cAMP (Europe-Finner & Newell, 1987). A model for chemosensory transduction has been proposed in which the activated cAMP and folate receptors interact with a membrane phospholipase that hydrolyses PIP_2 to 1,2-diacylglycerol and 1,4,5-IP_3 (similar to the response in mammalian cells to growth

hormones). The intracellular messenger 1,4,5-IP_3 then mobilizes intracellular calcium stores and this in turn stimulates guanylate cyclase activity to produce cGMP. Recently, it has been shown that a Dictyostelium ras gene, mutated in an analogous position to oncogenic mammalian ras gene, caused a reduction in the level of cGMP in D. discoideum cells (Van Haastert et al., 1987) after cAMP or folate stimulation. This suggests a role for $p23^{ras}$ in the chemotactic signalling pathway of D. discoideum.

One purpose of this study was to investigate the supposition that $p23^{ras}$ binds guanine nucleotides. The other purpose of this investigation was to immunopurify the ras protein from D. discoideum because a purified preparatin of p23 could be used in membrane reconstitution experiments to directly study its function.

MATERIALS AND METHODS

I. MATERIALS

Bacteriological peptone and yeast extract were from Oxoid and Bacto-Agar was from Difco. Dulbecco's modified Eagle medium and fetal bovine serum were from Gibco. Triton X-100 was from Amersham. N-tetradecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (Z-14) was from Calbiochem. 5.0 μm pore size filters were from Nucleopore. Nitrocellulose (0.45 μm pore size) was from Schleicher and Schuell. All radioisotopes were from New England Nuclear. Formalin fixed Staphylococcus aureus (Staph) was from The Enzyme Centre. Rabbit anti-rat immunoglobulin G (RARIG) was from Cooper Biomedical. Goat anti-rabbit immunoglobulin G (GARIG) and GARAT alkaline phosphatase conjugates were from Kirkegaard and Perry Laboratories, respectively. The rat myeloma cell line Y3-AG1.2.2 (Y3) was from the American Type Culture Collection. Y13-259 purified by high pressure liquid chromatography (HPLC) was from Oncogene Science. Diethylaminoethyl (DEAE)-Sephacel was purchased as pre-swollen beads from Pharmacia. SDS (specially pure) was from BDH. Bio-Beads SM-2, Affi-Gel 10, acrylamide, and tetramethylethylenediamine were from Bio-Rad. Bis-acrylamide and XAR-5 film were from KODAK. All other chemicals were of reagent grade from Fisher Scientific or Sigma Chemical Co.

II. METHODS

A) Organisms and Culture Conditions

Dictyostelium discoideum wild-type strain V12-M2 was used throughout this study. Strain V12-M2 was grown in association with *Enterobacter aerogenes* on nutrient agar plates until clearing of the bacterial lawn was discernible (Sussman, 1966). The cells were harvested from growth plates using sterile phosphate buffer (20 mM KH_2PO_4, K_2HPO_4, pH 6.0) and then washed free of bacteria with four differential centrifugations (700 x g for 2 min).

Harvey murine sarcoma virus (HaMSV) transformed, nonproducer NIH-3T3 mouse fibroblasts were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum at 37°C.

B) Radiolabeling of D. discoideum cells

For the preparation of radiolabeled cell extracts, cells were labeled with ^{35}S-methionine at the outset of differentiation as described previously (Weeks et al., 1987).

C) Preparation of Cell-Free Extracts

Crude cytosol and membrane fractions were prepared by resuspending the washed *D. discoideum* cells to 1 x 10^8 cells per ml of Tris-salts (10mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$), containing 40 µg/ml leupeptin and 40 µg/ml antipain. The cells were broken by passing them through a 5.0 µm pore size polycarbonate filter 2 to 3 times, as described by Das and Henderson (1983), until over 99% of the cells had been broken as assessed by microscopy. The lysate was centrifuged at

100,000 x g for 1 h, at 4°C to separate a crude cytosol (supernate) and a crude membrane (pellet) fraction. The 0.3% Triton X-100 membrane extracts were routinely prepared by resuspending the crude membrane fraction in an equal volume of Tris-salts plus 40 µg/ml leupeptin, 40 µg/ml antipain, and 0.3% (v/v) Triton X-100. This suspension was incubated for 15 min on ice, and then centrifuged at 100,000 x g for 1 h, at 4°C. Membrane extracts containing 1% Triton and 0.5% SDS were prepared for immunoaffinity chromatography using the same procedure except that the crude membrane fraction was resuspended in Tris-salts containing 40 µg/ml leupeptin, 40 µg/ml antipain, 1% (v/v) Triton X-100, and 0.5% (w/v) SDS.

Whole cell lysates were prepared for immunoprecipitation as described previously (Pawson et al., 1985) except the lysis buffer contained Tris-salts, 40 µg/ml leupeptin, 40 µg/ml antipain, and 1% (v/v) Triton X-100. The extracts were passed three times through an 18 gauge needle and particulate matter was removed by centrifugation at 100,000 x g for 1h, at 4°C. These lysates will be referred to as Triton-solubilized whole cell lysate throughout the remainder of this thesis.

HaMSV-transformed NIH-3T3 cells were harvested and lysed as described by Scolnick et al., (1979) except the cells were placed on ice after removal of the medium and washed with Tris-buffered saline. Cells were lysed by scraping them into 1 ml of Tris-salts, 40 µg/ml leupeptin, 40 µg/ml antipain, and 1% Triton X-100. The extracts were passed three times through a 23 gauge needle and clarified at 100,000 x g for 1 h, at 4°C.

D) Immunoprecipitation

All steps in this procedure were performed at 4°C. Cell lysates were adjusted to 1% Triton X-100 and 0.5% SDS. For the solubilization experiments (Figures 5, 6, and 7) particulate matter was removed by centrifugation at 15,000 x g for 1 h. Aliquots (0.5 ml) were precleared by an initial incubation for 15 min with 50 µl of a 1:10 suspension of Staph, followed by a second incubation with 50 µl of a 1:10 suspension of RARIG-coated Staph (RARIG-Staph) for 15 min. The Staph was pelleted by centrifugation at 13,000 x g for 2 min and the supernate was transferred to a new tube. Samples were incubated with 50 to 100 µl of ammonium sulphate-precipitated Y13-259 for 1 h. Control immunoprecipitations contained an equal volume of normal rat serum or ammonium sulphate-precipitated supernate (Jonak, 1980) from the Y13-259 parental myeloma, Y3. All samples were incubated with 50 µl of a 1:10 suspension of RARIG-Staph for 1 h. The immune complexes were pelleted by centrifugation at 13,000 x g for 1 min and the supernates were removed by aspiration. The immune complexes were resuspended by pipetting in 0.5 ml of Tris-salts containing 1% Triton X-100 and 0.5% SDS and washed by repeated centrifugation (3 to 5 times). Where indicated, SDS addition or preclearing were omitted.

E) Polyacrylamide Gel Electrophoresis

All immunoprecipitates and lysates were heated at 100°C in SDS sample buffer for 3 min unless otherwise indicated. All samples were electrophoresed through 11.25% or 12.5% SDS-polyacrylamide gels (Pawson et al., 1985).

F) Protein Determination

Protein was determined by the Folin method, as modified by Sandermann and Strominger (1972), or with the amido black protein assay (Schaffner & Weissman, 1973) if the protein concentration was less than 1 mg/ml.

G) Filter Assay for Guanine Nucleotide Binding

1) Guanosine 5'-Diphosphate (GDP) Binding. A 500 µl aliquot of Triton-solubilized whole cell lysate (2.36 mg) was incubated with 10^{-5} M ^{3}H-GDP (10 Ci/mmol) for 1 h at 4°C. The samples were immunoprecipitated directly without the addition of SDS or preclearing, using either normal rat serum of Y13-259 followed by incubation with RARIG-coated agarose beads (100 µl of a 1:10 suspension) for 1 h at 4°C. After washing, the immune complexes were collected on 0.45 µm pore size filters and washed three more times with 5 ml of ice-cold Tris-salts containing 1% Triton X-100. The amount of ^{3}H-GDP retained by the filters was determined by liquid scintillation counting. The purity of the ^{3}H-GDP was assessed by thin layer chromatography on poly-(ethyleneimine)-cellulose plates using 1.6 M LiCl in deionized H_2O as solvent (Randerath & Randerath, 1964). Radioactive spots were detected by exposing the chromatographed thin layer to XAR-5 film and quantitated by cutting the spots out and dissolving them in scintillation fluid for counting.

2) Guanosine 5'-Triphosphate (GTP) Binding. Aliquots (200) µl of Triton-solubilized whole cell lysate (0.79 mg) were precleared and then 10 µl of 40 µM S-γ-GTP and 2 µCi (2.7 µl) ^{35}S-γ-GTP were added to each sample. The samples were incubated

for 1h at 4°C and then directly immunoprecipitated with either normal rat serum or Y13-259 without addition of SDS. The immune complexes were washed and collected as described above.

Alternatively 600 µl aliquots of Triton-solubilized whole cell lysate (0.96 mg) were precleared and immunoprecipitated (in the absence of 0.5% SDS) with either normal rat serum or Y13-259. The immune complexes were washed and then resuspended in 200 µl of Tris-salts plus 1% Triton X-100. Two µM S-γ-GTP (10 µl of 40 µM stock) and 2.7 µl ^{35}-S-γ-GTP (2 µCi) were added to each aliquot and incubated for 1h at 4°C. The immune complexes were then collected on filters, washed, and counted as described above.

H) Western Blot Assay for Nucleotide Binding

Proteins were electroblotted from SDS-polyacrylamide gels to nitrocellulose as described by Towbin et al. (1979). Triton-solubilized whole cell lysate was immunoprecipitated in the absence of 0.5% SDS. The Triton-solubilized whole cell lysate samples and the immunoprecipitated samples were not heated before electrophoresis. The electrophoresed protein was electroblotted and probed as described previously for p21 ras (McGrath et al., 1984) except 50 µCi ^{35}S-γ-GTP was used in place of ^{32}P-α-GTP in 4 ml of binding buffer (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1% (v/v) NP40, 1 mg/ml bovine serum albumin). After incubation for 1h at 37°C in binding buffer, the electroblotted protein was washed for 10 min three times in 4 ml of binding buffer lacking bovine serum albumin (BSA) and GTP.

The dried filter was sprayed with En^{3}hance and was then subjected to fluorography at -70°C.

I) Western Immunoblot Assay for p23

Aliquots of 1% Triton X-100 membrane extracts (1.67 mg) were either directly electrophoresed, or immunoprecipitated with either Y13-259 or normal rat serum and then electrophoresed, through 12.5% SDS-polyacrylamide. The protein was electroblotted overnight at 30V in precooled transfer buffer (20 mM $NaPO_4$, pH 8.0). The Western blots were incubated in 15 ml of blocking buffer (3.0% (w/v) BSA, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 1 h at 37°C, on a gyratory incubator. The blot was then incubated in 15 ml of Y13-259 diluted 1/200 in blocking buffer, at 4°C, overnight. The filter was washed three times for 15 min, at room temperature with washing buffer (0.6% BSA, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl). RARIG (preadsorbed with methanol-fixed vegetative cells) was diluted 1/400 in 15 ml of blocking buffer and this was incubated with the filter for 1h at 37°C, shaking. The blot was then washed as described above and then incubated for 1h at 37°C on a gyratory shaker with GARIG conjugated to alkaline phosphatase diluted 1/3000 in 15 ml of blocking buffer. The blot was washed twice as before and once for 15 min in washing buffer without BSA. The blot was then incubated for 30 min at 37°C with 15 ml of developing solution (0.01 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 0.1 mg/ml nitroblue tetrazolium (NBT) in 1 M Tris-HCl, pH 8.8 containing 4 mM $MgCl_2$), rinsed twice with deionized H_2O and air-dried.

For the Western immunoblots and the Western blots used for guanine nucleotide binding, lanes containing size markers of known molecular weight were cut from the filter after electroblotting for staining with India Ink (Hancock & Tsang, 1983).

J) Photoaffinity Labeling

Photoaffinity labeling with crude cytosol and membrane fractions was conducted as described previously (Schleicher et al., 1986), with a few modifications. The membrane pellet was resuspended in Tris-salts containing 40 µg/ml leupeptin and 40 µg/ml antipain. The particulate matter was removed by centrifugation at 100,000 x g for 1h at 4°C before protein determination. Cell lysates were adjusted to 2 mg/ml protein and 50 µl aliquots were preincubated on ice with 5 µCi ^{32}P-α-GTP (750-800 Ci/mmol) in microtiter wells for 30 min. Irradiation with short-wave ultraviolet (UV) light (maximal emission, 254 nm) was for 60 min, at 4°C. After irradiation, samples were either mixed with SDS sample buffer and electrophoresed directly or immunoprecipitated with either Y3 or Y13-259 before electrophoresis through a 12.5% SDS-polyacrylamide gel. Size markers of known molecular weight were coelectrophoresed and their mobilities were determined by Coomassie blue staining. Gels were dried and autoradiographed with a screen at -70°C or -20°C.

In other experiments, Triton-solubilized whole cell lysate was immunoprecipitated before photoaffinity labeling with GTP. Triton-solubilized whole cell lysate was prepared in Tris-salts containing 40 µg/ml leupeptin, 40 µg/ml antipain, 1% Triton X-100, and 0.5% (w/v) SDS and 500 µl aliquots (3.34 mg protein)

were immunoprecipitated with either Y3 or Y13-259. The immune complexes were washed and then resuspended in Tris-salts. The immunoprecipitated samples were placed in microtiter wells for photoaffinity labeling with ^{32}P-α-GTP as described above. After irradiation, samples were mixed with SDS sample buffer and electrophoresed through a 12.5% polyacrylamide gel as described above.

To study the possible deleterious effect of photoaffinity labeling on immunoprecipitation, ^{35}S-methionine radiolabeled Triton-solubilized whole cell lysate was adjusted to 4 mg/ml protein and 0.5% Triton X-100. Aliquots (100 µg) were incubated with or without 1 mM GTP on ice for 30 min and irradiated, immunoprecipitated and electrophoresed as described above.

Nucleotide photoaffinity-labeling of HaMSV-transformed NIH-3T3 cell extract was performed as described by Stein et al. (1984) except for a few modifications. Aliquots (1 ml) were concentrated 10-fold with a Minicon B15 filter system at 4°C (about 2 h). After concentration, 40 µg leupeptin and 40 µg antipain were added and the sample was stored at -70°C. Each sample was preincubated with 1.2 µM ^{32}P-α-GTP (750-800 Ci/mmol) in microtiter wells for 1h in the dark, on ice. Irradiation with short-wave UV light was for 30 min at 4°C. After irradiation, samples were immunoprecipitated with either Y3 or HPLC-purified Y13-259 and electrophoresed as described above.
A 0.3% Triton X-100 membrane extract was prepared from D. discoideum vegetative cells and treated in the same manner as the transformed mammalian cell extract.

K) Enzyme-Linked Immunoadsorbent Assay (ELISA)

The standard ELISA was performed using a crude membrane fraction from vegetative cells as antigen. The protein was diluted to 0.1 mg/ml in carbonate buffer (14 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.02% (w/v) NaN_3, 5 mM $MgCl_2$, pH 9.6) and 100 µl was added to each well of a 96-well microtiter plate (except the substrate blank wells in the first row) for adsorption at 4°C, overnight. The wells were then washed three times with 0.3% BSA in phosphate-buffered saline (137 mM NaCl, 1.2 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 0.02% (w/v) NaN_3, pH 7.2) plus 0.05% (v/v) Tween 20 (PBS-Tween). Y13-259 ammonium-sulphate precipitated and control antibody (normal rat serum or Y3) were diluted 1/200 in PBS-Tween plus 0.3% BSA and 100 µl was added to the appropriate wells for incubation at 37°C for 1 h. No-antibody control wells contained PBS-Tween plus 0.3% BSA. Substrate blank controls that contained only substrate solution (p-nitrophenyl phosphate) were also included in each assay.

After the incubation, the wells were washed three times with PBS-Tween. RARIG (preadsorbed with vegetative cells) was diluted 1/2000 in PBS-Tween and 100 µl of this solution was added to each well (except substrate blank). The plates were incubated at 37°C for 1 h. The wells were washed three times with PBS-Tween and 100 µl of GARIG-alkaline phosphatase conjugate diluted 1/2000 in PBS-Tween was added to each well except the substrate blank. The ELISA plates were incubated for 1 h at 37°C and then washed three times with PBS-Tween. Finally,

100 µl of a 1 mg/ml solution of p-nitrophenyl phosphate dissolved in diethanolamine buffer (1 M diethanolamine, 0.02% NaN_3, 0.5 mM $MgCl_2$) was added to each well. After incubating with substrate at 37°C for 30 to 90 min, the $O.D._{405}$ of each well was taken using a Titertek. The substrate blank wells gave an average $O.D._{405}$ of 0.002 or less. The no-antibody control wells had an average $O.D._{405}$ that was 0.05 to 1.0 unit lower than the average $O.D._{405}$ obtained from the wells incubated with control antibody (Y3 or normal rat serum). Any changes from the standard ELISA are noted in the text or the footnotes to the tables.

Vegetative cells for the preadsorption of RARIG were prepared by resuspending washed and pelleted V12-M2 cells in anhydrous absolute methanol at 4°C to 10^7 cells/ml. Cells were fixed by end-to-end rotation for 30 min at 4°C. The methanol was washed from the cells by repeated centrifugation (three times) at 700 x g for 2 min in PBS. The pelleted cells were then resuspended in RARIG (reconstituted according to manufacturer's instructions) to 10^8 cells/ml and the sera was adsorbed on the end-to-end rotator overnight at 4°C. The cells were pelleted at 700 x g for 5 min and the RARIG was removed to another tube for storage at -20°C.

L) Preparation of Antibodies

Ammonium sulphate-precipitated Y13-259 or Y3 was purified by ion exchange chromatography on a DEAE-Sephacel column as described previously (Fahey & Terry, 1973). Immunoglobulin G

(IgG) was detected in fractions eluted from the column using RARIG in an Ouchterlony test.

M) Removal of Triton X-100

Triton X-100 was removed from samples prior to the adsorption of the protein to the ELISA plate using Bio-Beads SM-2 using the batch procedure described by Holloway (1973). Removal of Triton X-100 by acetone precipitation was done by adding an equal volume of ice-cold acetone to the membrane extract and incubating on ice for 30 min. The samples were centrifuged at 13,000 x g for 5 min and the supernates were poured off. The precipitates were placed under vacuum in a centrifuge (Speed-Vac) for 30 min to remove all the acetone. Pellets were resuspended in Tris-salts containing 40 µg/ml leupeptin and 40 µg/ml antipain.

N) Immunoaffinity Chromatography

Affi-Gel 10 was coupled to DEAE-purified Y13-259 or Y3 in 0.1 M MOPS, pH 7.5, for 4 h at 4°C. Between 5 and 12 mg antibody was coupled per ml gel.

For the experiments utilizing detection of p23 by ELISA, 5 ml immunoaffinity columns were used. Columns were equilibrated with Tris-salts. The membrane extract was applied to the column and the flow-through was recycled 3 to 5 times. The column was washed and eluted as described in the Results. One ml fractions were eluted into tubes containing 40 µg leupeptin and 40 µg antipain. The fractions were dialysed against 1 l of Tris-salts plus 100 µg/ml p-aminobenzamidine overnight at 4°C

and the protein content was determined. Either 10 µg of protein or 100 µl of each fraction was used as antigen for the ELISA and was adsorbed in buffer adjusted to pH of approximately 10 units.

For experiments utilizing the detection of p23 by immunoprecipitatin of radiolabeled membrane extracts, columns of 1 ml bed volume were used. The columns were equilibrated with the buffers described in the Results. Membrane extracts were prepared from 5 x 10^8 ^{35}S-methionine labeled cells and applied to the columns in a volume of 1 ml. The flow-through was recycled 3 to 5 times. Total protein content was estimated by trichloroacetic acid (TCA) precipitation of 5 µl aliquots that had been dried on glass fiber filters. The filters were washed with 20% TCA, 5% TCA and methanol, respectively, and then the radioactivity was determined by liquid scintillation in a Triton X-100 based scintillation fluid. The columns were washed with different detergent-containing Tris-salts buffers until all the TCA-precipitable radioactivity had been eluted. Each wash buffer was concentrated to 1 ml. The elution conditions that were used depended on the experiment and are described in the footnotes to tables. Eluted fractions were dialysed against 2 l of Tris-salts plus 100 µg/ml p-aminobenzamidine overnight at 4°C unless they contained Tris-salts already. The samples were split into two 0.5 ml aliquots and were immunoprecipitated with either Y13-259 or Y3. The immunoprecipitated proteins were electrophoresed through a 12.5% SDS-polyacrylamide gel.

Following treatment with En^{3}hance, the dried gel was exposed against XAR-5 film for one to six weeks. The p23 bands were quantitated by densitometry and these values were used to evaluate recovery of p23 from the columns.

O) Dot Blot

The amount of p23 in vegetative membrane preparations was also determined by the dot blot method described by Jahn et al. (1984) except the blots were incubated in either Y13-259 or normal rat serum diluted 1/100 or 1/200 for 1 h at 37°C or overnight at 4°C. After washing and reblocking the nitrocellulose sheets, they were incubated with RARIG (preadsorbed with vegetative cells) diluted 1/400 for 1 h. After washing for 5 min five times, the blots were incubated with GARIG-alkaline phosphatase conjugate that had been diluted 1/3000 in blocking solution containing 0.1% (v/v) Triton X-100 for 1 h. The blots were washed four times for 5 min in Tris/NaCl/0.1% Triton X-100 and then four times for 25 min in Tris/NaCl/0.1% Triton X-100. The blots were incubated for 30 min at 37°C in 10 ml of 1 M Tris-HCl, pH 8.8 containing 4 mg/ml $MgCl_2$, 0.01 mg/ml BCIP and 0.1 mg/ml NBT. The blots were rinsed with deionized H_2O and air-dried. DEAE-purified normal rat serum and different dilutions of RARIG were used unsuccessfully to try to lower the background.

RESULTS

SECTION I. GUANINE NUCLEOTIDE BINDING

The purified ras protein from mammalian cells ($p21^{ras}$) has a binding constant of 8×10^{-9} M for GTP and 7×10^{-9} M for GDP (Shih et al., 1980). Using at least ten times higher concentrations of guanine nucleotide, a number of different approaches were used to try to detect guanine nucleotide binding by $p23^{ras}$.

A) Filter assay

A previous study had shown that 100 µg of a cell-free lysate of Kirsten murine sarcoma virus (KiMSV) transformed NIH-3T3 cells will bind 1.10 pmoles of ^{3}H-GDP when immunoprecipitated with anti-p21 antibody whereas only 0.05 pmoles of ^{3}H-GDP are bound using control serum (Scolnick et al., 1979). In a filter assay using similar conditions, $p23^{ras}$ did not bind GDP (Table 1). The level of bound GDP in cell lysates immunoprecipitated with the anti-ras antibody was identical to that in cell lysates immunoprecipitated by normal rat serum. Using RARIG-Staph instead of RARIG-coated agarose for immunoprecipitation did not change the result and adding SDS to the immunoprecipitation increased the background levels of GDP bound with both specific antibody and normal rat serum (data not shown). The ^{3}H-GDP used in these experiments was determined to be 93 to 96 per cent pure by thin layer chromatography therefore the low amount of GDP binding was not due to degraded or contaminated GDP.

Table 1. Guanine Nucleotide Binding Filter Assay

Antibody	^{3}H-GDP bound[a] (fmol/mg)	^{35}S-γ-GTP bound (pmol/mg)[b]	
		Incubate First	Immunoprecipitate First
Normal rat serum	21.8 ± 9.2	1.93 ± 0.32	12.45 ± 4.47
Y-13-259	16.8 ± 13.2	1.92 ± 0.29	13.21 ± 6.69

a. The means and standard deviations of two determinations are given.

b. The numbers are the means of four determinations ± the standard deviation. "Incubate First" indicates GTP binding followed by immunoprecipitation and "Immunoprecipitate First" indicates immunoprecipitation followed by GTP binding.

Filter assays using the nonhydrolyzable GTP-analogue, S-γ-GTP, revealed higher levels of binding than those obtained with GDP but there was no indication of any specific binding to p23 ras (Table 1). When extracts were enriched for p23 by immunoprecipitating first and then incubating with GTP, the overall amount of GTP bound was increased but there was no indication of specific binding to the protein immunoprecipitated with Y13-259 (Table 1).

B) Western Blot Probed with GTP

When proteins were separated by SDS electrophoresis and then electroblotted onto nitrocellulose, several of the blotted proteins bound ^{35}S-γ-GTP. However, there was no labeling in the region that corresponded to $p23^{ras}$. The number of GTP-binding proteins was decreased if the lysates were first immunoprecipitated prior to electrophoresis, but the same GTP-binding proteins were immunoprecipitated by anti-ras antibody and by normal rat serum (Fig. 1). Again, there was no indication of GTP binding to a protein of the molecular weight of $p23^{ras}$.

C) Photoaffinity Labelling with GTP

Nucleotides can be covalently cross-linked to nucleotide-binding proteins using photochemical methods. Under the direct action of ultraviolet light, the binding of the ligand requires active enzyme and only occurs at the ligand-binding site on the protein (Yue & Schimmel, 1977). This technique has been used to detect several GTP-binding proteins in D. discoideum, including a strongly binding protein of M_r of 24,000 (Schleicher

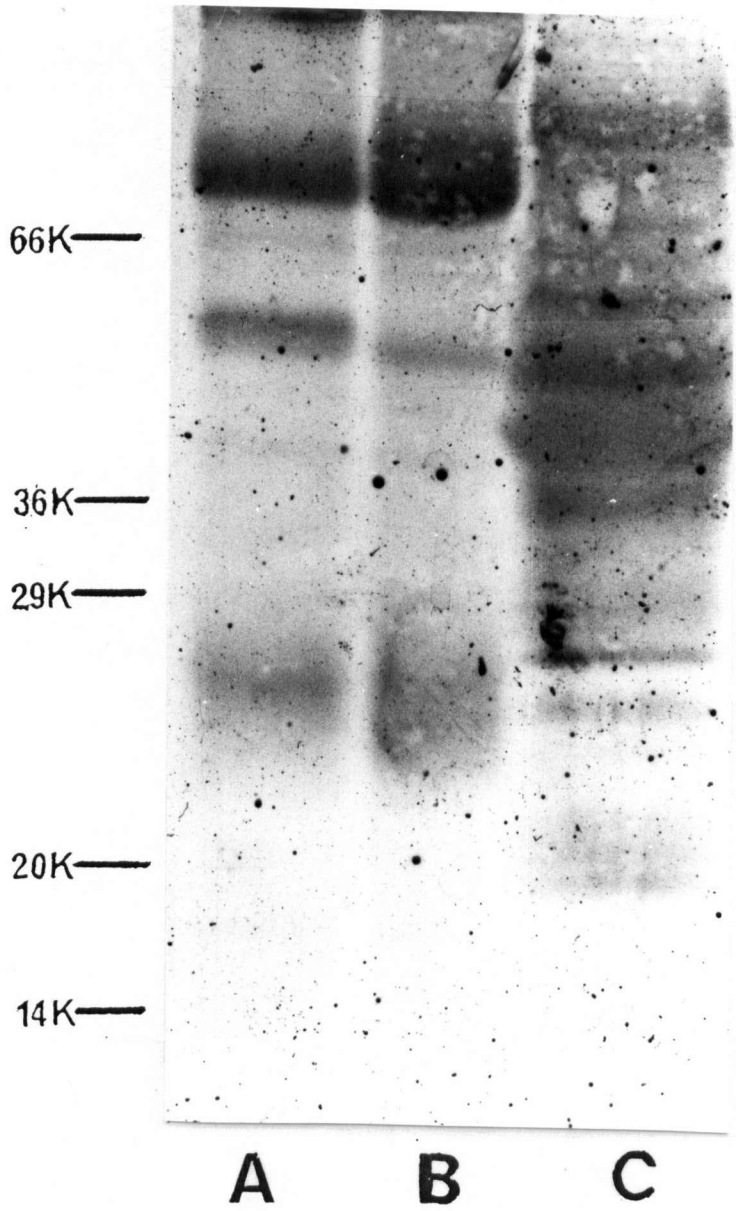

Figure 1. GTP Binding to D. discoideum Proteins. Samples were electrophoresed through an 11.25% polyacrylamide gel and the protein was transferred to nitrocellulose. The blot was probed with 50 μCi ^{35}S-γ-GTP as described in Methods. Two samples of Triton-solubilized whole cell lysate (1.2 mg protein) were treated with normal rat serum (Lane A) or Y13-259 (Lane B) and the immunoprecipitated material was subjected to electrophoresis. Lane C: 120 μg total protein. Molecular weight markers were visualized by the India Ink staining method.

et al., 1986). In order to determine whether this GTP-binding protein was actually the $p23^{ras}$, extracts were photoaffinity labeled with GTP and then either subjected to SDS electrophoresis directly or immunoprecipitated and then subjected to SDS electrophoresis. The resulting gels were dried and autoradiographed. A number of proteins in crude cytosol and membrane fractions bound GTP, including a protein of M_r about 24,000 (Fig. 2) but none of these proteins were immunoprecipitated with either the specific Yl3-259) or the control antibody (Y3). These results indicate that the $p23^{ras}$ protein does not bind GTP under these conditions and that the previously identified 24,000 M_r GTP-binding protein is not the ras protein. To enrich for $p23^{ras}$ in this assay, samples of Triton-solubilized whole cell lysate (3.34 mg protein) were immunoprecipitated with either control or Yl3-259 first and then the immunoprecipitated proteins were photoaffinity labeled and electrophoresed (see Methods). The results obtained were the same as those when $p23^{ras}$ was photoaffinity labeled and then immunoprecipitated.

Although Yl3-259 neutralizes $p21^{ras}$ activity, it does not interfere with the binding of GDP (Hattori et al., 1987) or GTP (Lacal & Aaronson, 1986). However, it is possible that GTP binding prevented the immunoprecipitation of $p23^{ras}$ in these experiments. To address this question, ^{35}S-methionine labeled Triton-solubilized whole cell lysate was photoaffinity labeled in the presence and absence of 1 mM GTP and then immunoprecipitated with control or anti-ras antibody (Fig. 3). In both

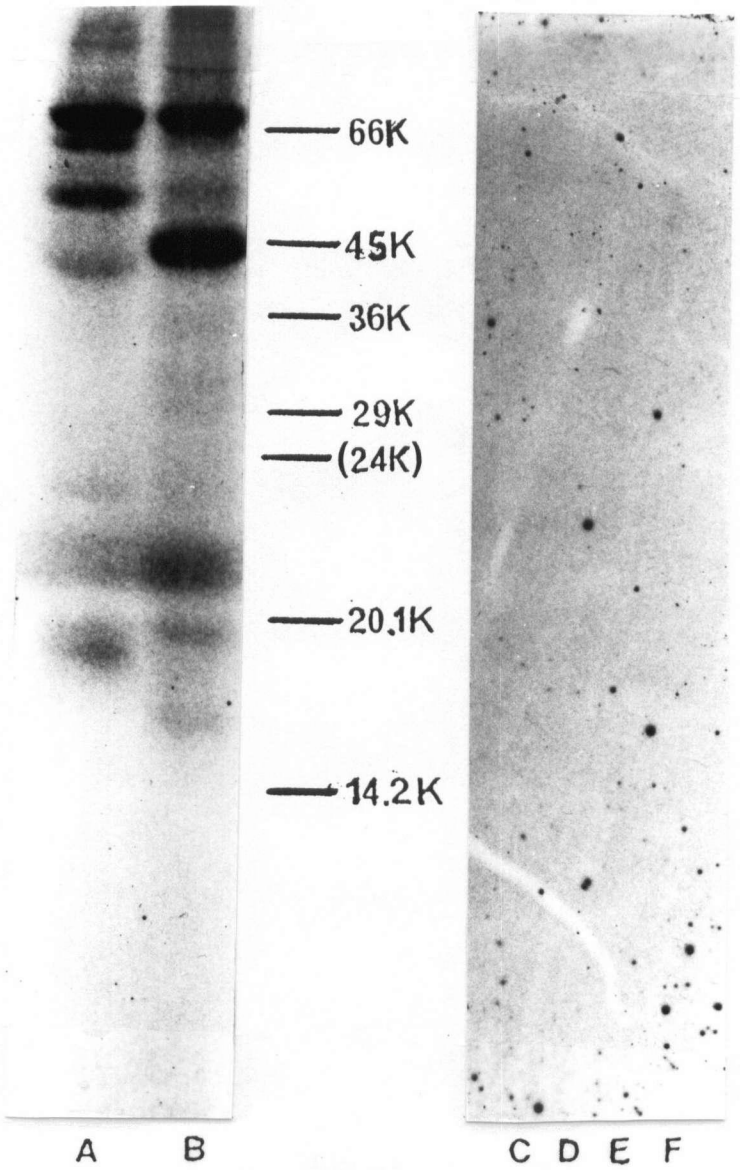

Figure 2. Direct Photoaffinity Labeling with GTP. Three aliquots each of 100 µg protein from crude cytosol and membrane fractions were incubated in the dark, on ice, with 1.4 x 10^{-7} M ^{32}P-α-GTP for 30 min. The samples were then irradiated with short wave UV light for 60 min. One cytosol (Lane A) and one membrane (Lane B) sample was mixed with SDS sample buffer and stored at -20°C while two samples each of cytosol (Lanes C and D) and membrane (Lanes E and F) proteins were immunoprecipitated with either control antibody (Lanes C and E) or Y13-259 (Lanes D and F). All samples were then subjected to electrophoresis through a 12.5% polyacrylamide gel. The gel was cut to separate Lanes A and B from Lanes C through F so that the dried gels could be exposed to film (with a screen and at -70°C) for different periods of time. Lanes A and B: exposed for 23 h. Lanes C through F: exposed for one week.

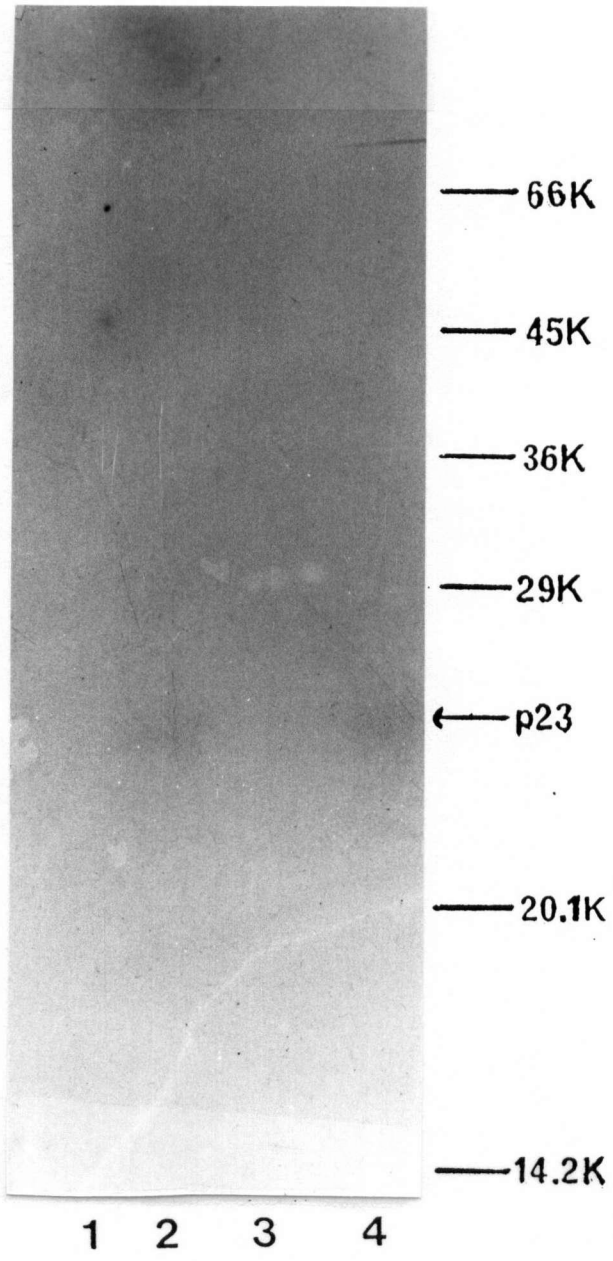

Figure 3. Photoactivation and Immunoprecipitation in the Presence and Absence of 1 mM GTP. Equal amounts (100 µg protein) of ^{35}S-methionine labeled Triton-solubilized whole cell lysate were incubated in the presence (1) and absence (2) of 1 mM GTP in the dark, on ice, for 30 min. The samples were then irradiated with short wave UV light for 60 min. Samples were then immunoprecipitated with either control (Lanes A and C) or anti-ras (Lanes B and D) antibody and electrophoresed through a 12.5% polyacrylamide gel as described in Methods. The dried gel was exposed to XAR-5 film for 10 days.

cases the p23 protein was immunoprecipitated by the Y13-259 antibody suggesting that GTP binding does not interfere with immunoprecipitation.

A positive control for GTP binding was provided by photoaffinity labeling a lysate from HaMSV-transformed rat cells and then immunoprecipitating with either anti-ras (Y13-259) or control antibody (Y3). The immunoprecipitates were then electrophoresed. The $p21^{ras}$ protein did bind GTP under these conditions (Fig. 4). A 0.3% Triton X-100 D. discoideum membrane extract was photoaffinity labeled under the same conditions and then immunoprecipitated with either Y13-259 or Y3 antibody. The immunoprecipitated proteins were then electrophoresed and autoradiographed. There is no indication of GTP binding by $p23^{ras}$ under these conditions.

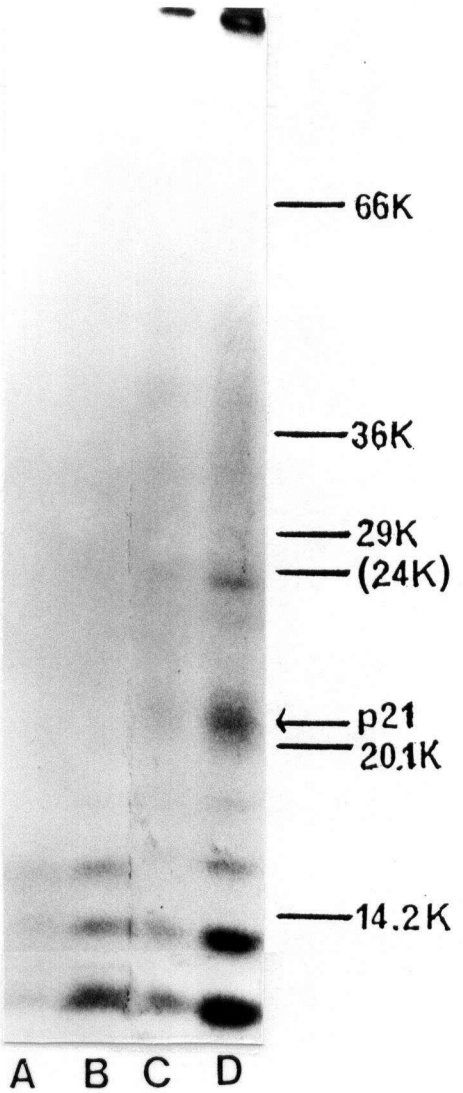

Figure 4. GTP Binding to $p21^{ras}$ from Harvey Murine Sarcoma Virus (HaMSV) Transformed NIH-3T3 Cells. Equal amounts of protein (2.5 mg in about 1 ml volume) from either a 0.3% Triton X-100 D. discoideum membrane extract (Lanes A and B) or a lysate of HaMSV-transformed NIH-3T3 cells (Lanes C and D) were concentrated to 100 µl at 4°C. Two 50 µl aliquots of each sample were incubated in microtiter wells with 1.2 µM ^{32}P-α-GTP in the dark for 60 min, on ice. The samples were then irradiated with short-wave UV light for 30 min. The photoaffinity-labeled samples were then immunoprecipitated with either 5 µl of Y3 (Lanes A and C) or 5 µl of HPLC-purified Y13-259 (Lanes B and D). The immunoprecipitated proteins were electrophoresed through a 12.5% SDS-polyacrylamide gel. The dried gel was exposed to film at -20°C with a screen for 4 days.

SECTION II. ATTEMPTED PURIFICATION OF $p23^{ras}$

Attempts were made to purify $p23^{ras}$ since a partially purified preparation should provide more definitive results in a GTP-binding assay in that other GTP-binding proteins should be removed and there should be relatively more p23 protein. Ultimately purified $p23^{ras}$ could be used in membrane reconstitution experiments to study the function of the protein. Immunoaffinity chromatography was chosen because it potentially provides a one-step purification procedure, the immunoprecipitation with Y13-259 works well, and there is an unlimited supply of the monoclonal antibody.

A) Solubilization Conditions

The $p23^{ras}$ is exclusively found in the crude membrane fraction rather than the cytosolic fraction after high-speed centrifugation in the presence of the protease inhibitors leupeptin and antipain (Weeks & Pawson, 1987). The p23 protein was removed from the membrane by Triton X-100 (Fig. 5), a concentration of 0.3% (w/v) being required for total solubilization (Fig. 6). Triton X-100 solubilization resulted in an enrichment of p23 of approximately four-fold over Triton-solubilized whole cell lysates.

The $p23^{ras}$ was not solubilized from the membrane by high pH, high salt, or the presence of EDTA (Fig. 7), suggesting that p23 is tightly bound to the membrane bilayer. In fact, $p23^{ras}$ is acylated (Weeks et al., 1987) like the mammalian *ras* protein, and the acyl group may anchor the protein to the membrane.

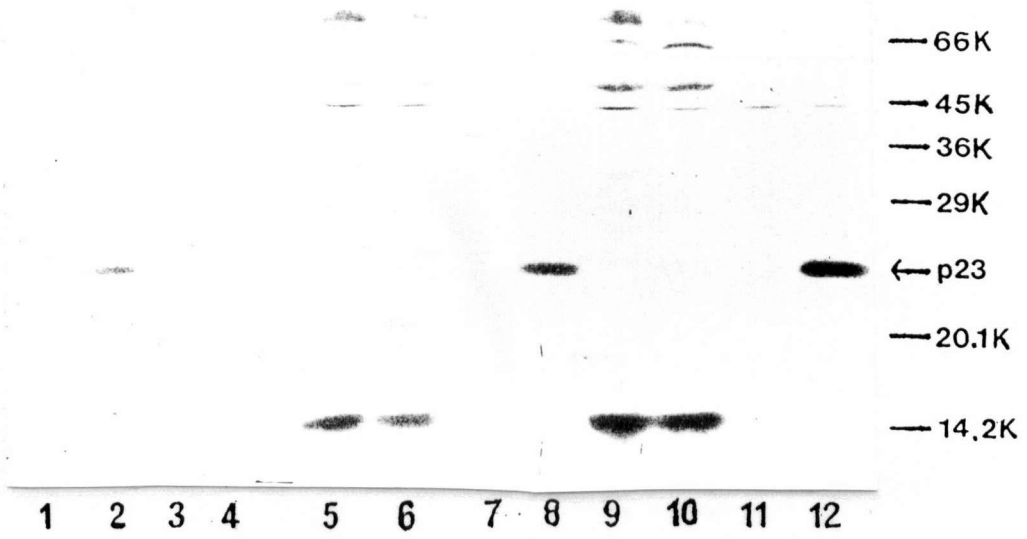

Figure 5. Solubilization of p23 with Triton X-100. Equal amounts of ^{35}S-methionine-labeled crude membranes were resuspended in 1 ml of Tris-salts containing 40 µg leupeptin, 40 µg antipain, and 0% (Lanes 1 to 4), 0.3% (Lanes 5 to 8), or 1.0% (Lanes 9 to 12) Triton X-100 and mixed well by vortexing. After a 15 min incubation at 4°C, the samples were recentrifuged to separate unsolubilized pellet (Lanes 1, 2, 5, 6, 9, 10) and solubilized supernatant (Lanes 3, 4, 7, 8, 11, 12). The pellets were then resuspended in 1 ml of buffer and each sample was split into two 0.5 ml aliquots for immunoprecipitation with 75 µl of either normal rat serum (Lanes 1, 3, 5, 7, 9, 11) or Y13-259 Lanes 2, 4, 6, 8, 10, 12). The immunoprecipitated protein was electrophoresed as described in Methods and fluorographed for 9 days.

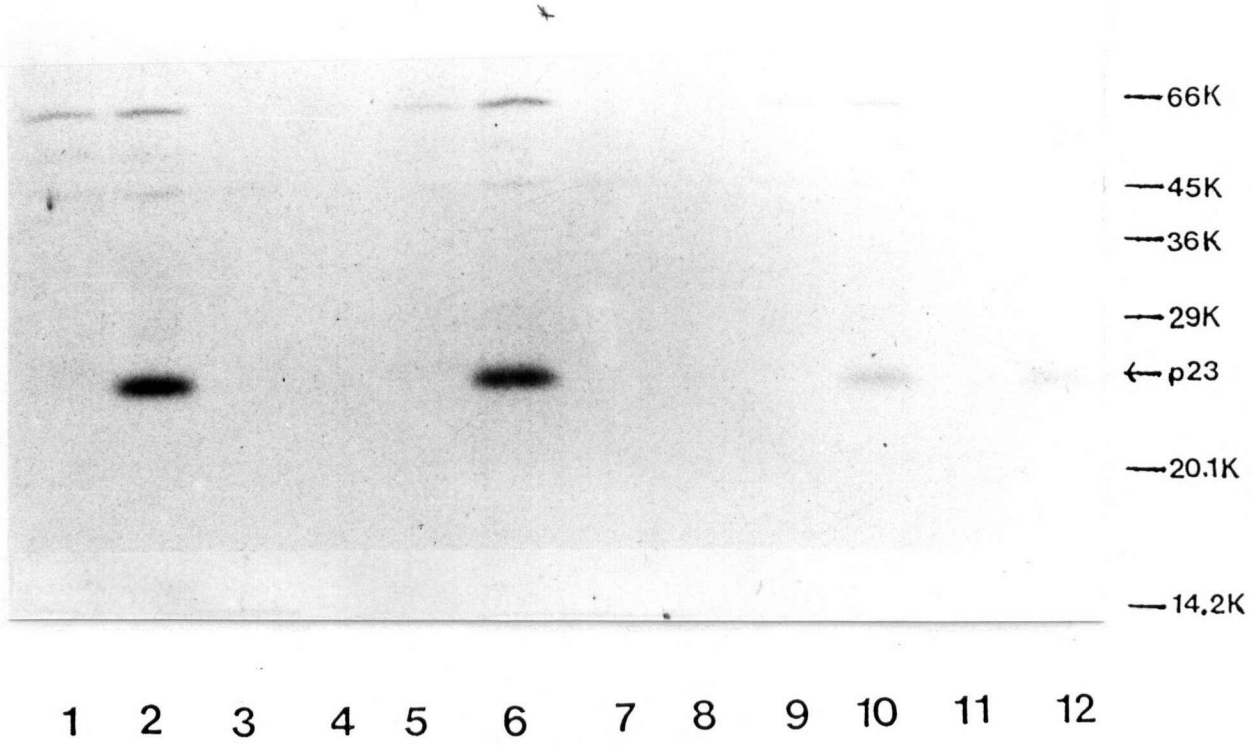

Figure 6. Effect of Low Concentrations of Triton X-100 on the Solubilization of p23. Equal amounts of radiolabeled crude membrane fractions were resuspended in 1 ml of Tris-salts containing 40 µg leupeptin, 40 µg antipain, and 0.01% (Lanes 1 to 4), 0.03% (Lanes 5 to 8), or 0.1% (Lanes 9 to 12) Triton X-100. After a 15 min incubation, the samples were recentrifuged to separate unsolubilized pellet (Lanes 1, 2, 5, 6, 9, 10) and solubilized supernatant (Lanes 3, 4, 7, 8, 11, 12). The pellets were resuspended in 1 ml of buffer and each sample was split into two 0.5 ml aliquots for immunoprecipitation with either normal rat serum (Lanes 1, 3, 5, 7, 9, 11) or Y13-259 (Lanes 2, 4, 6, 8, 10, 12). The immunoprecipitated protein was electrophoresed and fluorographed for 4 days.

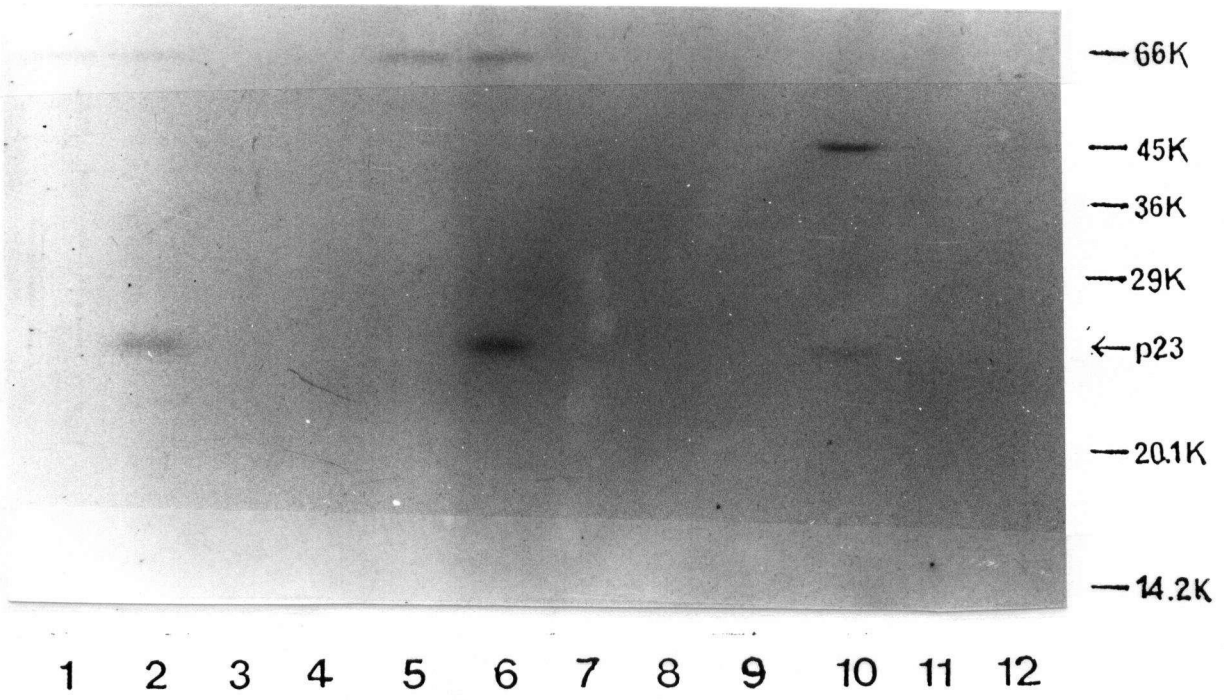

Figure 7. Effect of High Salt, Ethylenediamine Tetraacetic Acid (EDTA), or High pH on Solubilization of p23. Equal amounts of radiolabeled crude membranes were resuspended in 1 ml of Tris-salts containing 40 µg leupeptin, 40 µg antipain, and 0.5 M NaCl (Lanes 1 to 4), 10 mM EDTA (Lanes 5 to 8), or 100 mM Na_2CO_3 (Lanes 9 to 12). After a 15 min incubation, the samples were recentrifuged to separate unsolubilized pellet (Lanes 1, 2, 5, 6, 9, 10) and solubilized supernatant (Lanes 3, 4, 7, 8, 11, 12). The supernatants were dialysed against Tris-salts overnight at 4°C and the pellets were resuspended in 1 ml of buffer and stored overnight at -70°C. Each sample was split into two 0.5 ml aliquots and immunoprecipitated with either normal rat serum (Lanes 1, 3, 5, 7, 9, 11) or Y13-259 (Lanes 2, 4, 6, 8, 10, 12). The immunoprecipitated protein was electrophoresed and fluorographed for 4 days.

B) Detection by ELISA

The ELISA is a rapid and simple method that can be used to monitor protein purification. An assay was developed using vegetative membranes as the antigen, as described under Methods. In an attempt to lower backgrounds, a variety of modifications were tried: BSA, sheep IgG (Table 2), or 1% SDS (data not shown) as blocking agents, the use of DEAE-purified Y13-259, and the use of enzyme-antibody conjugate that had been first adsorbed with vegetative cells (data not shown). The best results were obtained with 0.3% BSA as blocking agent with Y3 as control antibody (Table 2). In addition, varying the incubation times did not improve the activity over background.

A two-step ELISA, using goat anti-rat IgG (GARAT) conjugated to alkaline phosphatase as the second antibody, gave high activity to control ratios but only at very high concentrations of GARAT (Table 3). For example, using Y3 as the control antibody, a 1/100 dilution of GARAT gave an activity to control ratio of 3.3 whereas the standard assay using the alkaline phosphatase conjugate at a dilution of 1/2000 gave an activity to control ratio of 1.7 (Table 2). However, the two-step ELISA proved to be cost-prohibitive due to the high concentrations of GARAT required for a high activity to control ratio.

In the standard ELISA, concentrations of Triton X-100 in excess of 0.003% reduce the Y13-259 activity to the same level as the control activity (data not shown). Other detergents (polyoxyethylene (20) sorbitan monolaurate (Tween 20), 3-(3-cholamidopropyl)dimethyl-ammonio (CHAPS), Z-14, polyoxethylene

Table 2. ELISA Using Vegetative Membranes as Antigen

Condition	O.D.$_{405}$ Normal Rat Serum	Y3	Y13-259	Activity - Control	A/C Ratio[a]
0.3% BSA	0.495 ± 0.028	N/D[b]	0.710 ± 0.024	0.215 ± 0.037	1.4
0.3% BSA	N/D	0.426 ± 0.037	0.710 ± 0.024	0.284 ± 0.044	1.7
0.001% Sheep IgG	0.374 ± 0.021	N/D	0.535 ± 0.023	0.161 ± 0.031	1.4
0.003% Sheep IgG	0.417 ± 0.033	N/D	0.510 ± 0.020	0.093 ± 0.0.39	1.2

a. Activity to control ratio.

b. Not determined.
The means and standard deviations of eight determinations are given. These results are from a single experiment but are representative of several experiments. Sheep IgG was added in place of BSA. The incubation with substrate was for 60 min.

Table 3. Two-Step ELISA with Goat Anti-Rat Immunoglobulin G (GARAT) Conjugated to Alkaline Phosphatase

Dilution of GARAT	O.D.$_{405}$ Normal Rat Serum	Y3	Y13-259	Activity - Control	A/C Ratio
1/20[a]	0.086 ± 0.014	N/D	0.736 ± 0.074	0.650 ± 0.075	8.6
1/100[b]	N/D	0.097 ± 0.003	0.319 ± 0.007	0.222 ± 0.008	3.3

a. The means and standard deviations of seven determinations are given. The incubation with substrate was for 30 min.

b. The means and standard deviations of two determinations are given. The incubation with substrate was for 90 min.

23-lauryl ether (Brij-35) have the same deleterious effect and n-octyl-glucoside gives a false positive result (data not shown). Removal of Triton X-100 with Bio-Beads SM-2 does not remove sufficient detergent to allow activity above control levels (data not shown). Acetone precipitation of samples did remove the Triton X-100 but resulted in only a very weak signal (Table 4).

Despite these problems, I tried to detect $p23^{ras}$ in fractions separated by immunoaffinity chromatography with the ELISA because I thought the purified protein would give a strong enough signal to be detected with this assay. Samples containing Triton X-100 were acetone-precipitated before being used for the ELISA.

A 0.3% Triton X-100 membrane extract was applied to a column that had been pre-equilibrated in Tris-salts (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl). In order to remove unbound protein and detergent the column was washed with Tris-salts until the $O.D._{280}$ reached zero. Several conditions were used to try to elute $p23^{ras}$: 0.5 M NaCl; 0.1 N HCl; 0.1 M glycine-HCl, pH 2.5 containing 50% ethylene glycol; 1 M and 4 M guanidine-HCl; and 3 M and 6 M NaSCN. In all experiments, $p23^{ras}$ was bound to the column but was not eluted by any of the elution conditions. Approximately 25% of the total protein applied to the column was never recovered. This suggested that Triton X-100 was needed during chromatography to keep the membrane proteins solubilized. An assay was therefore required

Table 4. ELISA with Vegetative Membranes and Acetone-Precipitated 0.3% Triton X-100 Membrane Extract

	O.D.$_{405}$			
Condition	Normal Rat Serum	Y13-259	Activity - Control	A/C Ratio
Vegetative Membranes	0.464 ± 0.070	0.872 ± 0.055	0.408 ± 0.089	1.9
Acetone-Precipitated	0.110 ± 0.014	0.192 ± 0.113	0.082 ± 0.114	1.7

The means and standard deviations of four determinations are given. The RARIG was diluted 1/1000 for this experiment and the incubation with substrate was for 30 min.

that could be used in the presence of detergent, since the ELISA had poor sensitivity on acetone-treated fractions.

Two immunoassays that can be performed with the antigen in the presence of detergent are the dot blot and the Western blot. The dot blot assay was insufficiently specific to be of value in that there was an equally strong activity when the antigen was incubated with Y13-259 antibody as when it was incubated with normal rat serum. The Western immunoblotting technique would only detect $p23^{ras}$ if the protein was immunoprecipitated prior to electrophoresis and then electroblotted to nitrocellulose for detection, and this method proved to be insufficiently sensitive (data not shown). The most sensitive assay that could be performed with the antigen in the presence of detergent was immunoprecipitation of radioactively-labeled proteins followed by radioautography. This assay was therefore used to monitor the fractionation of membrane extracts by immunoaffinity chromatography.

C) Detection by Immunoprecipitation

For these experiments, ^{35}S-methionine-labeled membrane extracts were applied to immunoaffinity columns that had been pre-equilibrated under various detergent conditions. Since the immunoprecipitation of p23 with Y13-259 is done in the presence of 1% Triton and 0.5% SDS, an immunoaffinity column equilibrated with the same detergents was initially used to try to purify p23. The membrane extract applied to this column was also solubilized with 1% Triton X-100 and 0.5% SDS. About 30%

of the total protein bound nonspecifically to this column but $p23^{ras}$ did not bind under these conditions (Table 5) so other buffer conditions were tried.

A membrane extract solubilized with 0.3% Triton X-100 was applied to a Y13-259 column that had been equilibrated in Tris-salts buffer (Table 6). Under these conditions there was little nonspecific binding by total protein but again none of the $p23^{ras}$ was bound.

Table 5. Immunoaffinity Chromatography with Y13-259 Column Equilibrated in Tris-Salts Plus 1% Triton X-100 and 0.5% Sodium Dodecyl Sulphate (SDS).

	Flow-Through	Elution 1[a]	Elution 2[b]	Not Recovered
Protein (%)	68	0.8	0.2	31
$p23^{ras}$ (%)	100	0	0	0

a. The column was eluted sequentially with 0.1 M diethylamine, 1 M guanidine, and 1 M NaSCN, each containing 1% Triton X-100.

b. The column was then eluted with the harsher condition of 6 M NaSCN + 1% Triton X-100.

Table 6. Immunoaffinity Chromatography with Y13-259 Column Equilibrated in Tris-Salts

	Flow-Through	Elution 1[a]	Elution 2[b]	Not Recovered
Protein (%)	98	1.7	0.3	0
$p23^{ras}$ (%)	100	0	0	0

a. The column was eluted sequentially with Tris-Salts + 0.5% SDS, 0.1 N HCl, and 0.1 M diethylamine each containing 1% Triton X-100.

b. The column was then sequentially eluted with 4M guanidine and 6 M NaSCN, each containing 1% Triton X-100.

DISCUSSION

In this study, I attempted to demonstrate GTP binding by $p23^{ras}$ using three different approaches: a filter assay, a Western blot assay, and photoaffinity labeling. The filter assay involved incubation of cell extracts with radiolabeled GDP or GTP followed by immunoprecipitation with anti-*ras* antibody. The immunoprecipitated complexes were then collected on nitrocellulose filters and counted directly. This approach has been used successfully to demonstrate GDP and GTP binding by KiMSV transformed cell lysates immunoprecipitated with anti-p21 antibody (Scolnick et al., 1979). Experiments where the immune complexes were boiled to release the protein instead of being collected on nitrocellulose filters showed guanine nucleotide binding by yeast cell extracts (Tamanoi et al., 1984). Since the same monoclonal antibody (Y13-259) specifically immunoprecipitates p21 and $p23^{ras}$ (Pawson et al., 1985), this assay should detect guanine nucleotide binding with *D. discoideum* cell extracts if p23 does bind guanine nucleotides. One possible reason for the absence of specific GTP binding by $p23^{ras}$ in the filter assays described in this investigation is the hydrolysis of bound GTP to GDP and its subsequent release by the enzyme. However, since $p23^{ras}$ did not bind a nonhydrolyzable GTP-analogue, this explanation was rejected.

For the Western blot assay proteins from cell lysates and immunoprecipitated protein were separated on polyacrylamide gels and then electro-blotted to nitrocellulose. The

nitro-cellulose was then incubated with radiolabeled GTP. This method had been used to show GTP binding by $p21^{ras}$ that had been expressed in Escherichia coli under conditions where this protein represented 5 to 10 per cent of the total intracellular bacterial protein (McGrath et al., 1984). In vegetative cells of D. discoideum, $p23^{ras}$ represents only 0.02 to 0.03 per cent of total protein (Pawson et al., 1985). The low cellular level of $p23^{ras}$ may account for the failure of the Western blot assay to detect GTP binding to the protein.

In the third approach, D. discoideum cell lysates were photoaffinity labeled with GTP and the labeled proteins were either electrophoresed directly or immunoprecipitated and then electrophoresed. In addition, cell lysates were immunoprecipitated and then photoaffinity labeled with GTP prior to electrophoresis. This approach led to detectable labeling of the $p21^{ras}$ protein from HaMSV-transformed NIH-3T3 cells in the presence of 1.2 μM GTP (Fig. 4), but $p23^{ras}$ exhibited no detectable GTP binding using the same experimental conditions.

One possible reason for a lack of GTP binding by cell lysates photoaffinity labeled and then immunoprecipitated is that the bound GTP interferes with immunoprecipitation of p23 by Y13-259. However, incubation of Triton-solubilized whole cell lysate with 1 mM GTP under similar conditions to those used in the photoaffinity labeling experiments did not interfere with immunoprecipitation of p23 by Y13-259 (Fig. 3).

There are two possible explanations for the lack of GDP/GTP binding by $p23^{ras}$: the protein from D. discoideum may

have a very low affinity for guanine nucleotides and binding is therefore undetectable using the experimental procedures described in this thesis or $p23^{ras}$ may not bind guanine nucleotides at all. The first possibility could theoretically be tested by using high concentrations of GTP but this experiment is impractical in terms of the large amounts of radiolabel that would be required. The second possibility seems unlikely in view of the fact that the RAS1 and RAS2 proteins of Saccharomyces cerevisiae bind GTP and GDP specifically (Temeles et al., 1985; Tamanoi et al., 1984) like the mammalian ras proteins (Scolnick et al., 1979; Shih et al., 1980; Trahey et al., 1987) although they apparently perform different physiological functions (Beckner et al., 1985; Toda et al., 1985; Uno et al., 1985).

A number of different enzyme activities in D. discoideum cells that had been transfected with the missense mutation Dd-ras-Thr^{12} have been examined (Van Haaster et al., 1987). This mutation, analogous to that found in oncogenic mammalian ras genes (Newbold, 1984), results in abberant morphogenesis of D. discoideum cells (Reymond et al., 1986). The Dd-ras-Thr^{12} transformants exhibit normal folate and cAMP-induced activation and desensitization of adenylate cyclase but the transient increase in cGMP is terminated earlier in these cells than in untransformed cells. This latter phenomenon is not due to a lower activity of guanylate cyclase or to a higher activity of cGMP-stimulated phosphodiesterase, but rather to an altered desensitization of guanylate cyclase. Thus $p23^{ras}$ does not

appear to be involved in the regulation of adenylate cyclase but to be directly or indirectly involved in the enhanced desensitization of guanylate cyclase.

The Dd-ras gene used in the studies described above was originally detected as a homologue of the mammalian ras gene (Reymond et al., 1984). It encodes a protein of M_r of 24,000. There are distinct differences in the expression of $p23^{ras}$ and the messenger ribonucleic acid (mRNA) that is hybridized by the ras gene during development. Synthesis of p23 is most rapid in vegetative cells and then drops during differentiation until the pseudoplasmodial stage where there is a slight increase in the rate of synthesis before the rate continues to drop to about 10% of the original ameoboid level at the end of development (Pawson et al., 1985). In contrast ras mRNA is present in low levels in vegetative cells, but in high levels in pseudoplasmodia (Reymond et al., 1984). At the pseudoplasmodial stage, newly synthesized p23 is localized in prespore cells (Weeks & Pawson, 1987) whereas p24 mRNA is localized in prestalk cells (Reymond et al., 1984). It is possible that $p23^{ras}$ and the product of the cloned ras gene are not identical and that each protein has a specific function at particular points of development. Thus $p23^{ras}$ may not be involved in the desensitization of guanylate cyclase like the product of the cloned ras gene and it may not act as a GTP binding regulatory protein since there is evidence from this study suggesting that it does not bind guanine nucleotides. However $p23^{ras}$ and

the cloned ras gene product may be identical in that the protein immunoprecipitated by Y13-259 is recognized by a polyclonal antibody raised against the ras gene product. Also Y13-259 thus far has been shown to immunoprecipitate all known ras gene products.

It is interesting to note that Firtel's group have not reported guanine nucleotide binding by the Dd-ras gene product and it is possible that they have attempted to demonstrate GTP binding, but have also been unsuccessful. Ras-related genes have been found in Drosopholia melanogaster (Weinberg & Shilo, 1981), in the mollusc Aplysia (Madaule & Axel, 1985), and Schizosaccharomyces pombe (Fukui & Kaziro, 1985), but there have been no reports that their gene products bind GTP or GDP.

The definitive determination of nucleotide binding by p23 awaits the purification of this protein. In addition, functional studies on the ras protein in D. discoideum will require purified protein. My attempts at purification of p23 by immunoaffinity chromatography have not been successful but I believe that this protein can at least be enriched by this method because other membranous proteins have been purified or greatly enriched using immunoaffinity chromatography, including the src oncogene product (Erikson et al., 1979).

There seems to be one main problem with the immunoaffinity purification of p23: $p23^{ras}$ does not bind to the column (Tables 5 and 6). The D. discoideum protein may be aggregating and this may prevent binding of $p23^{ras}$ to the antibody on the

column. Altering the equilibration and sample buffers may allow the disaggregation of the protein and allow the binding of $p23^{ras}$ to the column.

Membrane-associated proteins from other organisms have been purified by immunoaffinity chromatography in combination with other chromatographic methods (Aubry et al., 1987; Shen & Tai, 1986). This approach is difficult in the case of D. discoideum membrane proteins since they nonspecifically bind and aggregate very easily and do not fractionate easily on gel filtration and ion-exchange columns (MacDonald, 1986).

Other membrane proteins have been purified by passing the preparation over a column of the matrix itself (Hugues & August, 1982) or a column made with a nonspecific antibody (Suzuki et al., 1987) to remove nonspecifically binding protein before application to the immunoaffinity column. This approach was not necessary for this study because there was no problem with nonspecific binding if the column was equilibrated in the absence of detergent (Table 6).

Although it would be worthwhile to systematically vary the conditions of equilibration and binding in an attempt to obtain an enrichment of p23 by immunoaffinity chromatography, because the protein is found at such low levels in the cell, it may be simpler to isolate the genomic ras clone and express p23 in a suitable organism (such as E. coli) so that there is an enrichment for p23 before application to an immunoaffinity column. This would aid in the detection of the protein during

chromatography and should eliminate the problems of nonspecific aggregation by D. discoideum proteins.

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