MOLECULAR AND CELLULAR BIOLOGY, Jan. 1985, p. 33–39
0270-7306/85/010033-07$02.00/0

Vol. 5, No. 1

Regulation of a *ras*-Related Protein During Development of *Dictyostelium discoideum*

TONY PAWSON,[1] TERRY AMIEL,[1] EHLEEN HINZE,[1] NELLIE AUERSPERG,[2] NANCY NEAVE,[1] ANDRE SOBOLEWSKI,[1] AND GERALD WEEKS[1]*

Departments of Microbiology[1] and Anatomy,[2] University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5

Received 8 June 1984/Accepted 9 October 1984

Recent work has shown that DNA sequences related to the mammalian *ras* proto-oncogenes are highly conserved in eucaryotic evolution. A monoclonal antibody (Y13-259) to mammalian p21^{ras} specifically precipitated a 23,000-molecular-weight protein (p23) from lysates of *Dictyostelium discoideum* amoebae. Tryptic peptide analysis indicated that *D. discoideum* p23 was closely related in its primary structure to mammalian p21^{ras}. p23 was apparently derived by post-translational modification of a 24,000-molecular-weight primary gene product. The amount of p23 was highest in growing amoebae, but declined markedly with the onset of differentiation such that by fruiting body formation there was less than 10% of the amoeboid level. The rate of p23 synthesis dropped rapidly during aggregation, rose transiently during pseudoplasmodial formation, and then declined during the terminal stages of differentiation. There was, therefore, a strong correlation between the expression of the *ras*-related protein p23 and cell proliferation of *D. discoideum*.

The transforming sequences of acutely oncogenic RNA tumor viruses originate from genes present in normal vertebrate cells, termed proto-oncogenes (1). The effects of viral oncogene expression on cellular proliferation and differentiated phenotype suggest an active role for their normal cellular counterparts in cell growth and development. Some vertebrate oncogenes are transcribed at very different levels in distinct adult tissue types and during successive stages of mammalian embryogenesis (13, 14, 21, 25), implying that their functions are related to specific processes in vertebrate differentiation. In contrast, the *ras* proto-oncogenes are expressed at similar levels in many mammalian cell types (13, 14), and a developmental role is less obvious.

ras-related genes have been detected in the insect *Drosophila melanogaster* (24), and recently two genomic sequences closely related to mammalian *ras* genes have been defined in the budding yeast *Saccharomyces cerevisiae* (6). A broadly cross-reactive monoclonal antibody (Y13-259) originally raised against the p21^{ras} transforming protein of Harvey murine sarcoma virus (9) specifically recognizes a yeast protein which is presumably encoded by one of these yeast *ras* genes (16). This conservation of *ras* genes over such a wide range of evolution implies that they encode functions that are essential to all eucaryotic cells.

Dictyostelium discoideum is a simple eucaryotic organism in which the relationship of normal *ras*-encoded proteins to cellular proliferation and differentiation might be analyzed. It is unusual among eucaryotic organisms in that the processes of cell division and differentiation are largely separate. In the presence of nutrients, *D. discoideum* amoebae proliferate rapidly and are mutually indifferent, but upon starvation, cell division terminates, and cells aggregate. The aggregate forms a migrating pseudoplasmodium within which individual cells eventually differentiate into the spore or stalk cells of a mature fruiting body (11). There is only limited mitosis during differentiation, apparently restricted to prespore cells of the early pseudoplasmodial phase of differentiation (8, 32). We therefore examined *D. discoideum* for the presence of *ras*-related proteins with the intention of investigating their expression during *D. discoideum* development.

(A preliminary report of these data has been presented elsewhere [17a].)

MATERIALS AND METHODS

Growth of *D. discoideum*. The V12-M2 strain of *D. discoideum* was used throughout this study. Amoebae were grown in suspension cultures in association with *Escherichia coli* B23 as previously described (29). Briefly, bacteria were grown to stationary phase in M9 medium, pelleted by centrifugation at 5,000 × *g* for 10 min, and suspended in sterile phosphate buffer (20 mM KH_2PO_4 plus K_2HPO_4, pH 6.0) to an optical density at 660 nm of 6.0. The suspension was inoculated with *D. discoideum* amoebae (2×10^5/ml), and the amoebae were allowed to proliferate for 17 to 23 h at 22°C to a density of approximately 5×10^6/ml. To induce differentiation, amoebae were separated from bacteria by several low-speed centrifugations (700 × *g* for 2 min), and aliquots of 10^8 cells were plated on Millipore filters resting on Millipore support pads (26) saturated with sterile phosphate buffer containing 0.5 mg of streptomycin per ml. Kirsten murine sarcoma virus (KiMSV)-transformed, nonproducer NIH 3T3 mouse fibroblasts were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum at 37°C.

Radiolabeling of *D. discoideum* cells during growth. *D. discoideum* amoebae were labeled by growth on radioactive bacteria. Conditions were as described above except that the *E. coli* were grown in M9 medium supplemented with 2 mCi of [^{35}S]methionine per liter (1,000 Ci/mmol; Amersham Corp.), and *D. discoideum* was grown on the radioactive *E. coli*.

To examine levels of p23 during differentiation, radioactive *D. discoideum* cells were plated on Millipore filters as detailed above. Cells were washed from the filters with phosphate buffer after defined periods of differentiation, harvested by centrifugation, and suspended in lysis buffer (100 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-hydrochloride [pH

* Corresponding author.

7.5], 1% [vol/vol] Triton X-100, 0.5% [wt/vol] sodium dodecyl sulfate [SDS], 0.5 mM phenylmethylsulfonyl fluoride).

Radiolabeling of differentiating *D. discoideum* and mammalian cells. Nonradioactive *D. discoideum* cells were spread on Millipore filters as described above. At the appropriate times of differentiation, the filters were transferred on to a droplet containing 250 μCi of [^{35}S]methionine to initiate the labeling (7). At the end of the labeling period, cells were harvested from the filters into lysis buffer (30). KiMSV-transformed NIH 3T3 cells were labeled for 16 h with [^{35}S]methionine as described previously (30).

Immunoprecipitation. *D. discoideum* cells (10^8) were routinely lysed in 1 ml of ice-cold lysis buffer, and the cell lysates were clarified by centrifugation at 15,000 rpm for 30 min at 4°C. Aliquots (0.25 ml) of the resulting supernatants (approximately 5×10^6 trichloroacetic acid-precipitable cpm) were immunoprecipitated with Y13-259 anti-p21 rat monoclonal antibody as described in detail elsewhere (9, 30) except that the immunoprecipitates were washed with the previously described lysis buffer. To ensure that immunoprecipitation was complete, the supernatants from the precipitation were reincubated with anti-p21 antibody. Generally, little or no *ras*-related protein was precipitated by the second round of antibody. In most experiments, a rat monoclonal antibody specific to the transforming protein of Fujinami avian sarcoma virus (88AG) was used as a control. Radiolabeled KiMSV-transformed NIH 3T3 cells were lysed and immunoprecipitated with anti-p21 antibody by the same protocol.

Cell-free translation. Total cytoplasmic RNA was isolated from *D. discoideum* amoebae as described by Blumberg and Lodish (2) and translated in a messenger-dependent rabbit reticulocyte lysate prepared as described by Pelham and Jackson (18). The reticulocyte lysate was incubated with *D. discoideum* total cytoplasmic RNA at 0.5 mg/ml and [^{35}S]methionine at 2 mCi/ml for various periods of time at 30°C. For immunoprecipitation of the cell-free translation products, portions (0.05 to 0.1 ml) were mixed with 0.7 ml of lysis buffer on ice and immunoprecipitated with anti-p21 or control monoclonal antibodies. Polyadenylic acid selection of total RNA by affinity chromatography on oligodeoxythymidylic acid-cellulose, fractionation of polyadenylic acid-containing RNA by velocity centrifugation through a 15 to 30% glycerol density gradient, and cell-free translation of the size-fractionated RNA were performed as described previously (17) and as detailed in the legend to Fig. 5 (see below).

Gel electrophoresis. All immunoprecipitates were heated at 100°C in SDS sample buffer for 3 min and analyzed by electrophoresis through SDS-polyacrylamide gels (11.25% [wt/vol] acrylamide) (30). Gels were treated with En^{3}Hance (New England Nuclear Corp.) and exposed to preflashed XAR-5 (Kodak) X-ray film at −80°C. Size markers of known molecular weights were coelectrophoresed, and their mobilities were determined by Coomassie blue staining.

The amount of radioactivity in the *ras*-related protein bands was estimated by excision of the appropriate dried gel slice and scintillation counting in the presence of PCS scintillant. In some experiments, the amounts of radioactivity in the *ras*-related protein bands were determined by densitometry of the X-ray film.

Tryptic peptide analysis. [^{35}S]methionine-labeled proteins were eluted from polyacrylamide gels, oxidized, digested with tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin and analyzed as described previously (30). The two-dimensional separation of tryptic peptides on thin-layer cellulose plates employed electrophoresis at pH 2.1 in the first dimension followed by chromatography in *n*-butanol–acetic acid–water–pyridine (75:15:60:50) in the second dimension.

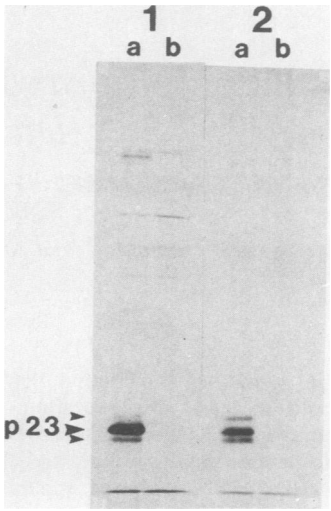

FIG. 1. Immunoprecipitation of *ras*-related proteins from *D. discoideum* amoebae and pseudoplasmodial cells. Detergent lysates of [^{35}S]methionine-labeled *D. discoideum* amoebae (lanes 1) or pseudoplasmodial *D. discoideum* cells (lanes 2) were subjected to immunoprecipitation with Y13-259 anti-p21 rat monoclonal antibody (a) or with a control rat immunoglobulin G monoclonal antibody (b). Amoebae were labeled by growth on radioactive *E. coli*, whereas the pseudoplasmodial cells were labeled directly with [^{35}S]methionine at between 14 and 16 h of differentiation. Immunoprecipitates were analyzed by electrophoresis on an 11.25% SDS-polyacrylamide gel, and radiolabeled proteins were detected by fluorography.

RESULTS

Identification of *D. discoideum* proteins antigenically related to p21^{ras}. *D. discoideum* amoebae (strain V12-M2) were radiolabeled by vegetative growth for 23 h on [^{35}S]methionine-labeled *E. coli*, lysed in a detergent buffer, and immunoprecipitated with Y13-259 anti-p21 monoclonal antibody (Fig. 1). A *D. discoideum* protein with a molecular weight of 23,000 (p23) was specifically precipitated by the antibody together with less intensely labeled polypeptides with mobilities of 24,000 (p24) and 22,000 (p22). No proteins were immunoprecipitated from lysates of *E. coli* alone (data not shown), indicating that p23 was not a contaminating bacterial protein. In a further experiment, *D. discoideum* amoebae were grown on nonradioactive *E. coli* and induced to differentiate by removal of the bacteria; after 14 h of differentiation, pseudoplasmodial cells were incubated with [^{35}S]methionine for 2 h, and the labeled cells were then immunoprecipitated with anti-p21 antibody. The same three proteins were specifically detected by the Y13-259 antibody in these radiolabeled differentiating cells (Fig. 1).

Structural relationship between *D. discoideum* p23 and mammalian p21^{ras}. The specific precipitation of *D. discoideum* p23 with anti-p21^{ras} antibody, taken together with its similarity in size to mammalian p21^{ras}, suggested that p23 might be the product of a *D. discoideum ras* gene and thus might be related in primary structure to mammalian p21^{ras}. We therefore compared *D. discoideum* p23 and the p21^{ras} protein encoded by the Ki-*ras* gene of KiMSV by using two-dimensional tryptic peptide mapping of immunoprecipitated [^{35}S]methionine-labeled proteins. This form of comparative tryptic peptide analysis is very sensitive to minor

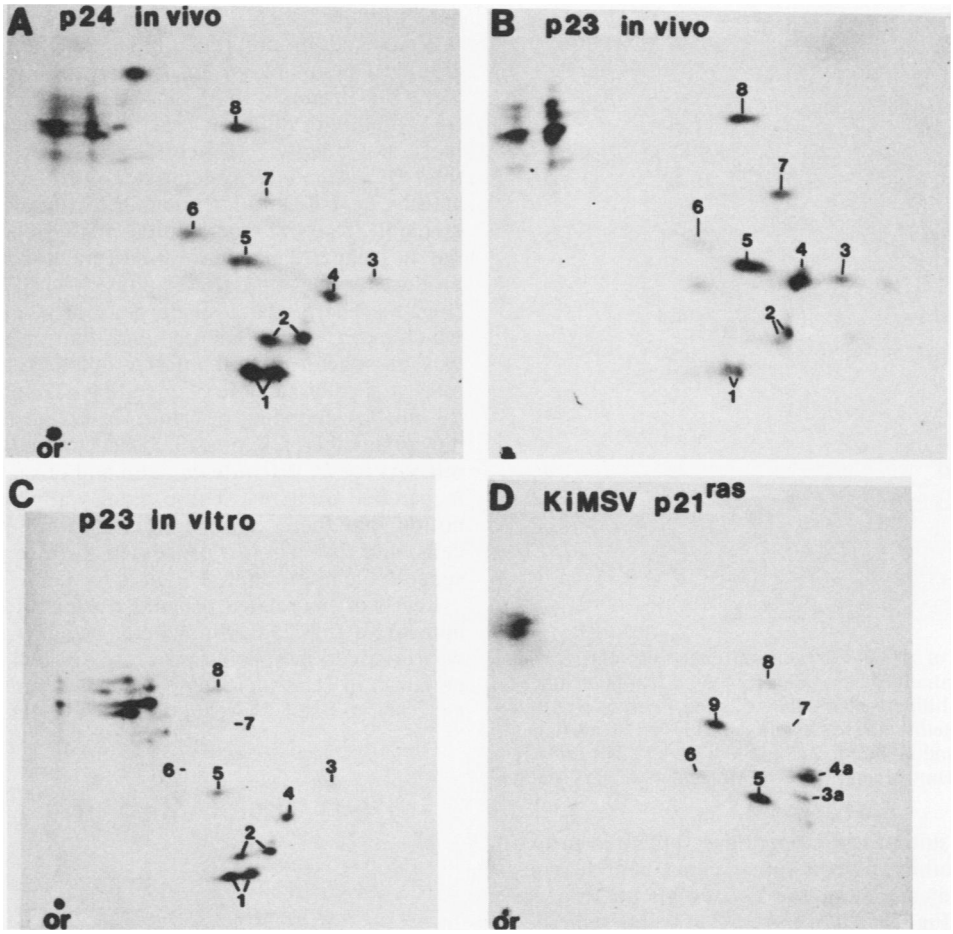

FIG. 2. Tryptic peptide analysis of *D. discoideum ras*-related proteins. [^{35}S]methionine-labeled proteins were immunoprecipitated with anti-p21 antibody from pulse-labeled differentiating *D. discoideum* cells from a messenger-dependent rabbit reticulocyte lysate programmed with *D. discoideum* RNA or from radiolabeled KiMSV-transformed NIH 3T3 mouse fibroblasts. Proteins were digested with trypsin and analyzed by two-dimensional tryptic peptide mapping. Each tryptic digest was spotted on a thin-layer cellulose plate (Or) and separated by electrophoresis at pH 2.1 in the first dimension (anode on the left, cathode on the right) and by chromatography in the second dimension (from bottom to top). Plates were coated with En^{3}Hance spray and exposed to XAR-5 film at −80°C. Comigrating peptides were given the same numbers; peptides 1 and 2 frequently migrated as doublets. A, p24 from labeled cells; B, p23 from labeled cells; C, p23 synthesized in the reticulocyte lysate; D, KiMSV p21^{ras} (nonphosphorylated form).

differences between two proteins since a single amino acid change can have a profound effect on the migration of a peptide, and changes of lysine or arginine residues or of the labeled amino acid can yield entirely new radiolabeled tryptic peptides. Nonetheless, the patterns of [^{35}S]methionine-labeled peptides obtained after two-dimensional separation of trypsin digests of p23 and p21^{ras} were very similar (Fig. 2). KiMSV p21^{ras} yields three strongly labeled methionine-containing tryptic peptides (Fig. 2D, spots 4a, 5, and 9) and four less-intense spots, some of which may represent differently modified forms of the major peptides (Fig. 2D, spots 3a and 6 to 8). *D. discoideum* p23 yielded four major (Fig. 2B, spots 1, 2, 4, and 5) and four minor (Fig. 2B, spots 3, 6, 7, and 8) methionine-containing tryptic peptides. Four of eight *D. discoideum* p23 peptides comigrated with peptides of KiMSV p21^{ras} (the major tryptic peptide [spot 5] and the minor spots 6 to 8). Peptide 5 was also found in tryptic digests of immunoprecipitated [^{35}S]methionie-labeled yeast *ras* protein (E. Hinze, unpublished data). In addition, tryptic peptides 3a and 4a of p21^{ras} had similar, although not identical, migrations to those of peptides 3 and 4 of p23. KiMSV p21^{ras} apparently lacked peptides 1 and 2 of p23 and possessed a distinct methionine-containing peptide (peptide 9) with no obvious relationship to any p23 tryptic peptide. These data suggested that p23 was closely related in structure to KiMSV p21^{ras} and indicated that p23 was a *ras* protein.

Synthesis of *D. discoideum* p23. *D. discoideum* grown in the presence of [^{35}S]methionine-labeled *E. coli* contains p23 as the major radiolabeled antigenically *ras*-related protein (Fig. 1, lane 1a) with barely detectable levels of p24. In contrast, when cells were incubated with [^{35}S]methionine for only 2 h, considerably more label was incorporated into p24 relative to p23 (Fig. 1, lane 2a). This observation suggested the possibility that p24 might be a precursor which is post-translationally modified to yield p23. This hypothesis was consistent with the data shown in Fig. 3, in which nonradioactive amoebae were plated on filters and then radiolabeled for 15 or 120 min before immunoprecipitation with anti-p21 antibody. During the 2-h labeling period, the kinetics of p24 and p23 labeling indicated a precursor-product relationship since the level of labeled p24 remained relatively constant, whereas the amount of labeled p23 increased markedly. To examine the structural relationship between p24 and p23, labeled proteins were excised from the gel and digested with

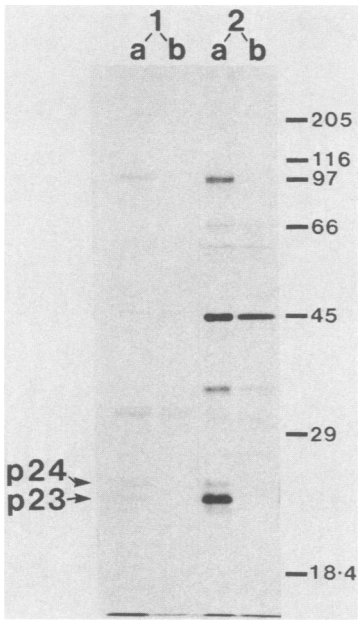

FIG. 3. Synthesis of *D. discoideum ras*-related proteins during the first 2 h of differentiation. Nonradioactive *D. discoideum* amoebae were plated on Millipore filters (10^8 cells per filter) and pulsed with 250 μCi of [^{35}S]methionine for 15 min (lanes 1) or 120 min (lanes 2). Cells were lysed and immunoprecipitated with Y13-259 anti-p21 antibody (a) or with control antibody (b). Molecular weights are in thousands.

trypsin, and the resulting tryptic peptides separated in two dimensions. The methionine-containing tryptic peptide maps of p24 and p23 indicated that the two polypeptides were virtually identical (Fig. 2). All of the labeled tryptic peptides contained within p23 were represented by comigrating peptides in the map of digested p24 as would be expected if p23 were derived from a p24 precursor.

In vitro translation and sizing of *D. discoideum ras*-related messenger RNA. To examine the nature of the primary gene product in more detail, total cytoplasmic RNA was extracted from *D. discoideum* amoebae and translated in a messenger-dependent rabbit reticulocyte lysate protein-synthesizing system. The translation products were immunoprecipitated with anti-p21 antibody (Fig. 4). Two *ras*-related proteins were synthesized in vitro corresponding in size to p23 and p24 from intact cells. A tryptic peptide map of p23 synthesized in the reticulocyte lysate was essentially identical to that of p23 from labeled cells (Fig. 2), indicating that the *ras*-related proteins identified by cell-free translation were the same as those immunoprecipitated from *D. discoideum* cell lysates. Since both p23 and p24 were detected by in vitro translation, we wished to determine their relative kinetics of synthesis. The reticulocyte lysate and *D. discoideum* RNA were therefore incubated for various times, and the products were analyzed by immunoprecipitation with anti-p21 antibody (Fig. 4, lanes 1 to 4). The larger protein was detected within 10 min, accumulated during the subsequent 60 min, and then remained constant throughout the remainder of the incubation. In contrast, p23 was not detectable until 30 min, but was the major component of immunoprecipitable material after 120 min. For comparison, *ras*-related proteins labeled in vivo by extended growth of amoebae on radioactive bacteria were displayed on the same gel, with p23 constituting the only strongly labeled band (Fig. 4, lane 6). These data argued that p24 was a primary *ras* gene product which was post-translationally processed both in cells and in the reticulocyte lysate to yield p23. An alternative explanation for these results is that p24 and p23 were the products of distinct genes whose mRNAs were translated with different kinetics. To investigate this possibility, polyadenylic acid-containing RNA was selected from total *D. discoideum* vegetative cell RNA and was fractionated by centrifugation through a 15 to 30% glycerol density gradient. The size-fractionated mRNAs were translated in the messenger-dependent rabbit reticulocyte lysate, and the products were immunoprecipitated with anti-p21 antibody (Fig. 5). There was a single peak of p24-encoding activity which exactly cosedimented with that of p23. Further analysis of vegetative cell mRNA failed to reveal any other mRNA species capable of encoding p23 and p24. The size of the mRNA encoding p24 and p23 was estimated to be 13S (approximately 1 kilobase), based on sedimentation of the mRNAs for *D. discoideum* actin and of rat tRNA and rRNA in parallel gradients. These data were consistent with the notion that there is a single mRNA for p24 in vegetative cells, and that p23 is a processed form of p24 rather than a separate gene product.

Levels of *ras*-related proteins during *D. discoideum* development. If this 23,000-molecular-weight *ras*-related protein was involved in specific processes of cell growth or differentiation in *D. discoideum*, it might be anticipated that p23 expression itself is regulated during the transition from

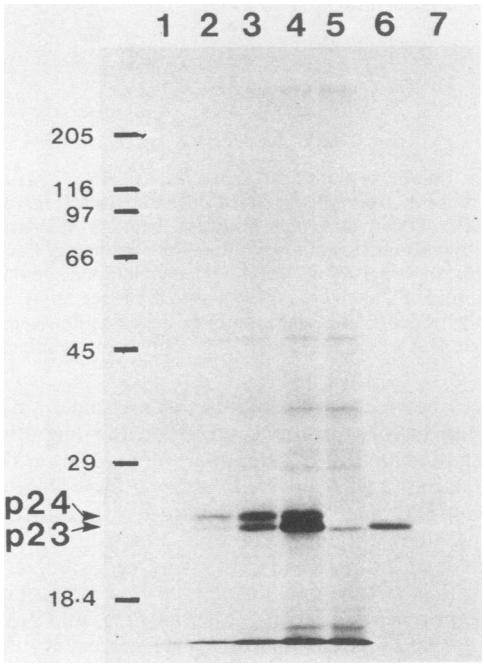

FIG. 4. Kinetics of *ras*-related protein synthesis determined with rabbit reticulocyte lysate. Total cell RNA from *D. discoideum* amoebae was added to a messenger-dependent rabbit reticulocyte lysate containing [^{35}S]methionine and incubated at 30°C. At 10 (lane 1), 30 (lane 2), 60 (lane 3), or 120 min (lanes 4 and 5) after the start of incubation, samples were removed and immunoprecipitated with anti-p21 antibody (lanes 1 to 4) or control antibody (lane 5). For comparison, *D. discoideum* amoebae grown for 17 h on [^{35}S]methionine-labeled *E. coli* were lysed and immunoprecipitated with anti-p21 antibody (lane 6) or control antibody (lane 7). The mobilities of size markers and their molecular weights in thousands are indicated.

vegetative growth to differentiation. We therefore grew *D. discoideum* amoebae for approximately six generations on [^{35}S]methionine-labeled *E. coli*, removed residual bacteria, and induced differentiation by spreading cells on Millipore filters in the absence of exogenous nutrients. At different times after the onset of differentiation, cells were lysed and immunoprecipitated with an excess of anti-p21 antibody. The amount of p23 dropped markedly during the 24-h period required for formation of the terminal cell types (Fig. 6). In this experiment, differentiation occurred in the complete absence of nutrients; under these conditions, all amino acids used for protein biosynthesis during differentiation are derived from degradation of preexisting protein synthesized during amoeboid growth (31). Thus, any de novo-synthesized p23 was labeled with [^{35}S]methionine derived from protein degradation, and the amount of label represented the absolute level of p23 in the cell. Since total cellular protein declines gradually during the course of differentiation as a consequence of endogenous respiration (31), the amounts of antigenically *ras*-related proteins (p22, p23, and p24) were normalized relative to total radiolabeled cell protein (Fig. 7). The levels of the *ras*-related proteins dropped approximately fourfold during the first 10 h of differentiation, remained relatively constant between 12 and 16 h, and then declined to less than 10% of the original value by the end of differentiation. Even at its highest level in vegetative cells p23 is a minor cellular constituent and represents only 0.02 to 0.03% of total radiolabeled cell protein as determined by the immunoprecipitation procedure.

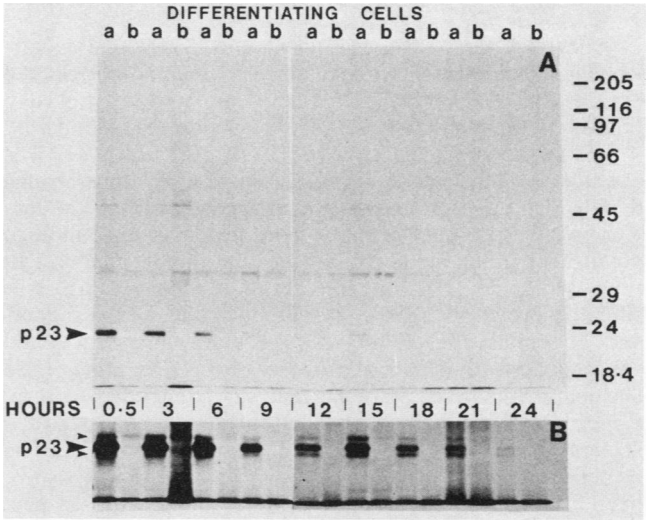

FIG. 6. Immunoprecipitation of *ras*-related proteins during the course of *D. discoideum* differentiation. *D. discoideum* amoebae that had been radiolabeled by growth on [^{35}S]methionine-labeled *E. coli* were allowed to differentiate for the indicated times, then harvested into lysis buffer, and immunoprecipitated with (a) Y13-259 anti-p21 rat monoclonal antibody or with (b) control rat monoclonal antibody. The immunoprecipitates were analyzed by electrophoresis on 11.25% SDS-polyacrylamide gels, and radiolabeled proteins were detected by fluorography by exposure to Kodak XAR-5 X-ray film for 3 days (A) or 4 weeks (B). The mobilities and molecular weights in thousands of a number of standard proteins are indicated.

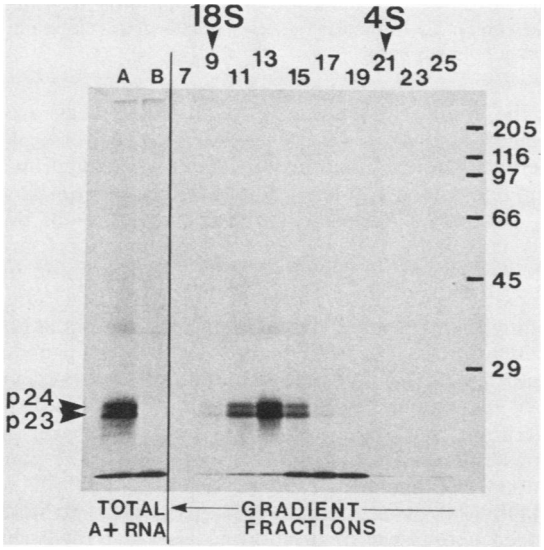

FIG. 5. Size fractionation of mRNAs encoding p24 and p23. Total *D. discoideum* vegetative cell RNA was polyadenylic acid selected (A+ RNA), translated in a messenger-dependent rabbit reticulocyte lysate, and immunoprecipitated with anti-p21 antibody (A) or with control antibody (B). In addition, 25 μg of selected A+ RNA was centrifuged on a 15 to 30% glycerol gradient (40,000 rpm, 10 h, 20°C, SW41 rotor), and the gradient was fractionated into 25 fractions. The sedimentations of 18S and 4S rat RNAs from a parallel gradient are indicated. RNA was recovered from the gradient fractions and translated in vitro, and the cell-free products were immunoprecipitated with anti-p21 antibody. In the experiment shown here, every other fraction was analyzed. Fraction numbers are indicated above the corresponding lanes. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography as described above.

Synthesis of *ras*-related proteins during differentiation. The rapid drop in the overall level of the antigenically *ras*-related proteins after the onset of differentiation might be explained by a complete cessation of synthesis. However, labeling of differentiating cells (Fig. 1) indicated that p23 was synthesized during the pseudoplasmodial stage of development. It was therefore pertinent to establish whether the rates of *ras*-related protein synthesis varied during the differentiation process. To determine a reliable labeling procedure with which to quantitate p23 synthesis, cells were incubated with [^{35}S]methionine after the onset of differentiation, and incorporation into p23 was assayed by immunoprecipitation with anti-p21 antibody. The results revealed that the incorporation of label into p23 was still increasing after 2 h of incubation (Fig. 3). Cells were therefore labeled with [^{35}S]methionine for 2-h periods during differentiation and were then immunoprecipitated with anti-p21 antibody. The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis, and the rate of specific *ras*-related protein synthesis relative to total cellular protein synthesis was calculated. The rate declined rapidly during the first 10 h of differentiation, increased between 10 and 14 h (the period that corresponded to tip and pseudoplasmodium formation), and then declined again towards the end of differentiation (Fig. 8). It is noteworthy that the level of p23 during differentiation determined under steady-state labeling conditions (Fig. 6 and 7) reflected the rate of p23 synthesis determined by short-term labeling experiments (Fig. 8). There was a striking correspondence between the transient elevation of specific *ras*-related protein synthesis and the interruption in the general decline of total p23 levels during pseudoplasmodium formation. It seems probable that the lowered rates of synthesis accounted at least in part for the overall drop in p23 levels during development.

DISCUSSION

D. discoideum cells synthesized a 23,000-molecular-weight protein which was specifically immunoprecipitated by an anti-p21^{ras} monoclonal antibody and was closely related to mammalian p21^{ras} in structure as defined by tryptic peptide analysis. Like p21^{ras}, *D. discoideum* p23 was a relatively minor cellular protein. Normal and transforming mammalian p21^{ras} proteins are synthesized as 22,000-molecular-weight (pro-p21) primary gene products and undergo post-translational modification to a 21,000-molecular-weight species (15, 20, 23). Apparently, *D. discoideum* p23 was similarly processed from a 24,000-molecular-weight precursor. The similarities in the size, structure, abundance, and apparent post-translational processing of *D. discoideum* p23 and mammalian p21^{ras} argued that p23 is encoded by a *D. discoideum ras* gene and provided further evidence that *ras* sequences are widely distributed in eucaryotic cells. The proteins encoded by the yeast *ras*^{sc}*1* and *ras*^{sc}*2* genes have been predicted to contain 309 and 322 amino acids, respectively, and a 42,000-molecular-weight protein has been tentatively identified as the product of the *ras*^{sc}*2* gene (19). The yeast *ras* proteins are therefore substantially larger than the 189-amino-acid protein p21^{ras}. Although a direct comparison between the primary structures of p23 and p21^{ras} must await isolation and nucleotide sequence determination of the *D. discoideum ras* gene(s), it was clear from the experiments with cell-free translation of amoeboid RNA that the primary gene product of *D. discoideum ras* had an apparent molecular weight of 24,000. It therefore seems probable that p23 lacks the additional sequences found in the yeast *ras* proteins.

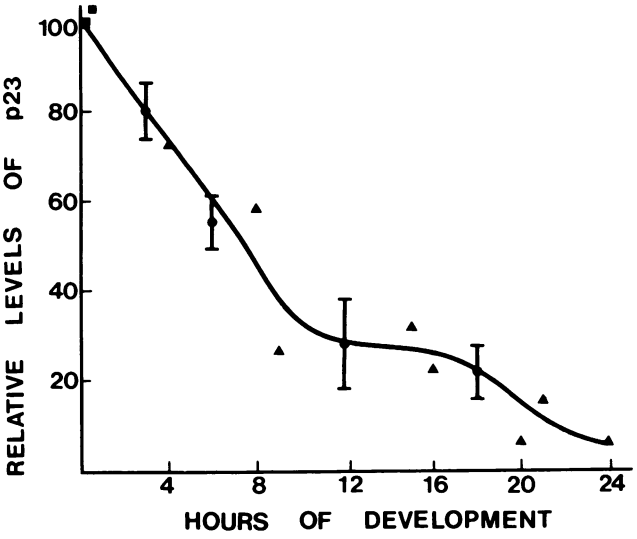

FIG. 7. Level of *ras*-related proteins relative to total cellular protein during *D. discoideum* differentiation. The radioactivity in the *ras*-related proteins immunoprecipitated from differentiating cells prelabeled by growth as amoebae on radioactive bacteria (see legend to Fig. 6) was determined by excision of the dried gel slices and liquid scintillation counting in PCS scintillant. Background counts were obtained from the control immunoprecipitations. The [^{35}S]methionine counts per minute in p24, p23, and p22 relative to total trichloroacetic acid-precipitable counts per minute in the original cell lysates were calculated. This relative value averaged 0.022% at the onset of differentiation. The plotted data are from three independent experiments normalized to 100% for time zero and represent either a single determination (▲), the mean of two determinations from separate experiments (●), or the mean of three separate determinations (■). The appearance of the tip, which marks the onset of pseudoplasmodium formation, occurred at 12 h in these experiments.

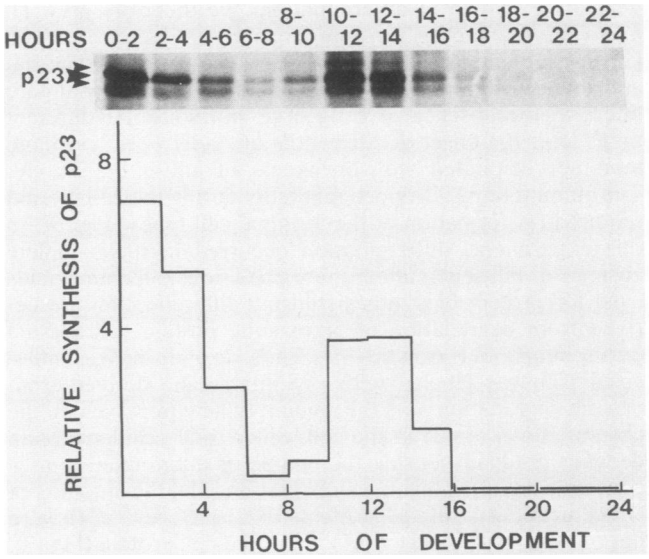

FIG. 8. Synthesis of *D. discoideum ras*-related proteins during differentiation. Differentiating *D. discoideum* cells (10^8) were labeled for the indicated 2-h periods with 250 μCi of [^{35}S]methionine. Cells were lysed and immunoprecipitated with Y13-259 anti-p21 antibody, and the immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis. The label incorporated into *ras*-related proteins (p24, p23, and p22) during these 2-h pulses was determined by optically scanning the resulting fluorograms and integrating the peaks. The total incorporation into protein was measured by trichloroacetic acid precipitation of the original cell lysate. The extent of *ras*-related protein synthesis for each 2-h period is expressed as a fraction of the total protein synthesis in arbitrary units. Tip formation occurred at 10 h of development in the experiment shown here.

There was a clear and reproducible down-regulation of *ras* protein expression and level during *D. discoideum* development, suggesting that the synthesis and levels of p23 are closely correlated with the extent of cellular proliferation. There was a transient elevation in p23 synthesis during the early pseudoplasmoidal phase of differentiation, and it is intriguing that the only detectable mitosis that occurs during differentiation is restricted to this same early pseudoplasmodial stage (8, 32). We postulate that in *D. discoideum* the level of *ras*-encoded proteins is an important factor in cell proliferation. This interpretation is consistent with the recent observation that disruption of both yeast *ras*^{sc} genes by insertional mutagenesis renders resulting haploid spores nonviable (28). It is interesting in this context to note that enhanced expression of the normal Ha-*ras-1* rat or human cellular genes under control of a retroviral long terminal repeat can induce the neoplastic proliferation of NIH 3T3 mouse fibroblasts, indicating that mammalian cell growth is sensitive to changes in p21^{ras} levels (4, 5). In addition, elevated levels of Ha-*ras* RNA are expressed in regenerating rat livers (10), and Ki-*ras* transcription in mouse BALB/c 3T3 fibroblasts is modulated in a cell cycle-dependent manner consistent with a role for *ras* genes in mitosis (3). In general, vertebrate *ras* gene expression as measured by RNA levels has shown little variation with differentiation, although moderate decreases in *ras* RNA have been reported during terminal differentiation of teratocarcinoma stem cells (3). This may result from the difficulty in separating proliferation and differentiation in complex mammalian

developmental systems. In addition, studies on mammalian *ras* expression have relied on measurements of specific RNA concentrations, which do not necessarily reflect the amounts of the corresponding proteins since regulation may be exerted at the translational or post-translation level.

Mammalian $p21^{ras}$ proteins bind guanine nucleotides (15), display GTPase activity (12), and show a familial relationship to the G proteins that regulate adenyl cyclase activity (12). The extraordinary structural conservation of *ras*-encoded proteins argues that their function is also retained during eucaryotic evolution. *D. discoideum*, by virtue of the simplicity of its developmental pathway, affords a model for investigating the mechanisms by which expression of *ras* proteins are modulated and for elucidating the nature of their biochemical activities. It will be intriguing to determine whether the down-regulation of p23 during *D. discoideum* development is an active and integral requirement for the process of cellular differentiation initiated by starvation or whether it simply represents a passive response to the cessation of cell division.

ACKNOWLEDGMENTS

We are grateful to M. Furth and E. Scolnick for the generous gift of Y13-259 anti-p21 monoclonal antibody and to K. Tatchell for communicating his results before publication.

This work was supported by grants from the National Cancer Institute of Canada, the Medical Research Council of Canada, and the British Columbia Health Care Research Foundation.

LITERATURE CITED

1. **Bishop, J. M.** 1983. Cellular oncogenes and retroviruses. Annu. Rev. Biochem. **52**:301–354.
2. **Blumberg, D. D., and H. F. Lodish.** 1980. Complexity of nuclear and polysomal RNAs in growing *Dictyostelium discoideum* cells. Dev. Biol. **78**:268–284.
3. **Campisi, J., H. E. Gray, A. B. Pardee, M. Dean, and G. E. Sonenshein.** 1984. Cell cycle control of c-*myc* but not c-*ras* expression is lost following chemical transformation. Cell **36**:241–247.
4. **Chang, E. H., M. E. Furth, E. M. Scolnick, and D. R. Lowy.** 1982. Tumorigenic transformation of mammalian cells induced by a normal human gene homologous to the oncogene of Harvey murine sarcoma virus. Nature (London) **297**:479–483.
5. **DeFeo, D., M. A. Gonda, H. A. Young, E. H. Chang, D. R. Lowy, E. M. Scolnick and R. W. Ellis.** 1981. Analysis of two divergent rat genomic clones homologous to the transforming gene of Harvey murine sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **78**:3328–3332.
6. **DeFeo-Jones, D., E. M. Scolnick, R. Koller, and R. Dhar.** 1983. *ras*-related gene sequences identified and isolated from *Saccharomyces cerevisiae*. Nature (London) **306**:707–709.
7. **Devine, K. M., J. H. Morrissey, and W. F. Loomis.** 1982. Differential synthesis of spore coat proteins in prestalk and prespore cells of *Dictyostelium*. Proc. Natl. Acad. Sci. U.S.A. **79**:7361–7365.
8. **Durston, A. J., and F. Volk.** 1978. The spatial pattern of DNA synthesis in *Dictyostelium discoideum* slugs. Exp. Cell Res. **115**:455–457.
9. **Furth, M. E., L. J. Davis, B. Fleurdelys, and E. M. Scolnick.** 1982. Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus and of the cellular *ras* gene family. J. Virol. **43**:294–304.
10. **Goyette, M., C. J. Petropoulos, P. R. Shank, and N. Fausto.** 1983. Expression of a cellular oncogene during liver regeneration. Science **219**:510–512.
11. **Loomis, W. F.** 1975. *Dictyostelium discoideum*: a developmental system. Academic Press, Inc., New York.
12. **McGrath, J. P., D. J. Capon, D. V. Goeddel, and A. D. Levinson.** 1984. Comparative biochemical properties of normal and activated human *ras* p21 protein. Nature (London) **310**:644–649.
13. **Muller, R., D. J. Slamon, E. Adamson, J. M. Tremblay, D. Muller, M. J. Cline, and I. M. Verma.** 1983. Transcription of c-*onc* genes c-ras^{Ki} and c-*fms* during mouse development. Mol. Cell. Biol. **3**:1062–1069.
14. **Muller, R., D. J. Slamon, J. M. Tremblay, M. J. Cline, and I. M. Verma.** 1982. Differential expression of cellular oncogenes during pre- and postnatal development of the mouse. Nature (London) **299**:640–644.
15. **Papageorge, A., D. Lowy, and E. M. Scolnick.** 1982. Comparative biochemical properties of p21 *ras* molecules coded for by viral and cellular *ras* genes. J. Virol. **44**:509–519.
16. **Papageorge, A. G., D. DeFeo-Jones, P. Robinson, G. Temeles, and E. M. Scolnick.** 1984. *Saccharomyces cerevisiae* synthesizes proteins related to the p21 gene product of *ras* genes found in mammals. Mol. Cell. Biol. **4**:23–29.
17. **Pawson, T., P. Mellon, P. Duesberg, and G. S. Martin.** 1980. *env* gene of Rous sarcoma virus: identification of the gene product by cell-free translation. J. Virol. **33**:993–1003.

17a. **Pawson, T., and G. Weeks.** 1984. Expression of *ras*-encoded proteins in relation to cell growth and differentiation, p. 461–470. *In* M. Bishop, J. Rowley, and M. Greaves (ed.), Genes and cancer. Alan R. Liss, Inc., New York.

18. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67**:247–256.
19. **Powers, S., T. Kataoka, O. Fasano, M. Goldfarb, J. Strathern, J. Broach, and M. Wigler.** 1984. Genes in *S. cerevisiae* encoding proteins with domains homologous to the mammalian *ras* proteins. Cell **36**:607–612.
20. **Sefton, B., I. S. Trowbridge, and J. A. Cooper.** 1982. The transforming proteins of Rous sarcoma virus, Harvey sarcoma virus and Abelson virus contain tightly bound lipid. Cell **31**:465–474.
21. **Shibuya, M., H. Hanafusa, and P. Balduzzi.** 1982. Cellular sequences related to three new *onc* genes of avian sarcoma viruses (*fps*, *yes*, and *ros*) and their expression in normal and transformed cells. J. Virol. **42**:143–152.
22. **Shih, T., P. E. Stokes, G. W. Smythers, R. Dhar, and S. Oroszlan.** 1982. Characterization of the phosphorylation sites and the surrounding amino acid sequences of the p21 transforming proteins coded for by the Harvey and Kirsten strains of Murine sarcoma viruses. J. Biol. Chem. **257**:11767–11773.
23. **Shih, T. Y., M. O. Weeks, P. Gruss, R. Dhar, S. Oroszlan, and E. M. Scolnick.** 1982. Identification of a precursor in the biosynthesis of the p21 transforming protein of Harvey murine sarcoma virus. J. Virol. **42**:253–261.
24. **Shilo, B.-Z., and R. A. Weinberg.** 1981. DNA sequences homologous to vertebrate oncogenes are conserved in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S.A. **78**:6789–6792.
25. **Sorge, L. K., B. T. Levy, and P. F. Maness.** 1984. $pp60^{c\text{-}src}$ is developmentally regulated in the neural retina. Cell **36**:249–257.
26. **Sussman, M.** 1966. Biochemical and genetic methods in the study of cellular slime mold development. Methods Cell. Physiol. **2**:397–410.
27. **Taparowsky, E., K. Shimizu, M. Goldfarb, and M. Wigler.** 1983. Structure and activation of the human N-*ras* gene. Cell **34**:581–586.
28. **Tatchell, K., D. T. Chalett, D. DeFeo-Jones, and E. M. Scolnick.** 1984. Requirement of either of a pair of *ras*-related genes of *Saccharomyces cerevisiae* for spore viability. Nature (London) **309**:523–527.
29. **Town, C. D., and J. Gross.** 1978. The role of cyclic nucleotides and cell agglomeration in postaggregative enzyme synthesis in *Dictyostelium discoideum*. Dev. Biol. **63**:412–420.
30. **Weinmaster, G., E. Hinze, and T. Pawson.** 1983. Mapping of multiple phosphorylation sites within the structural and catalytic domains of the Fujinami avian sarcoma virus transforming protein. J. Virol. **46**:29–41.
31. **White, G. J., and M. Sussman.** 1961. Metabolism of major cell components during slime mold morphogenesis. Biochim. Biophys. Acta **53**:285–293.
32. **Zada-Hames, I. M., and J. M. Ashworth.** 1978. The cell cycle and its relationship to development in *Dictyostelium discoideum*. Dev. Biol. **63**:307–320.