A *ras*-related gene from the lower eukaryote *Dictyostelium* that is highly conserved relative to the human *rap* genes

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ABSTRACT

The cellular slime mold *Dictyostelium discoideum* contains two *ras* genes, Dd*ras*G and Dd*ras* that are differentially expressed during development. We have characterized a gene that hybridized to both Dd*ras* and Dd*ras*G under low, but not under high stringency conditions. The deduced amino acid sequence is highly conserved with respect to the human *rap* (K*rev*-1, *smg*21) proteins and the corresponding gene has been designated Dd*rap*1. The Dd*rap*1 gene is expressed at all stages during development but is expressed maximally during the aggregation and culmination periods when the expression of Dd*ras* and Dd*ras*G is declining. During vegetative growth and early development Dd*rap*1 cDNA hybridizes to a single mRNA of 1.1 kb. As development progresses the level of this mRNA declines and messages of 1.0 and 1.3 kb appear.

INTRODUCTION

Over the past few years it has become evident that *ras* genes are part of a larger superfamily and members of this superfamily have been found in a wide variety of organisms. This widespread phylogenetic conservation suggests that they perform functions that are essential to all eukaryotic cells. The major sequence conservation within the superfamily is the preservation of four domains implicated in the binding of guanine nucleotides (1), but there are additional regions of conservation and the individual members of the *ras* gene family vary in the amount of this additional identity. The *ral*, R-*ras* and *rap* genes are more closely related to *ras* genes with approximately 50% amino acid identity, whereas the *rho* and *rab* genes have only approximately 30% identity (2). The conservation of the nucleotide binding domains suggests that the members of the *ras* gene superfamily are all guanine nucleotide binding proteins, presumably regulating some aspect of signal transduction.

The proteins encoded by the recently described human *rap* genes contain the four domains associated with guanine binding but have an unusual feature in that one of these highly conserved domains contains a threonine at residue 61 instead of the customary glutamine (3–5). This difference is of major interest since virtually any amino acid substitution at position 61 in the Ha-*ras* protein is correlated with an ability to transform cell lines (6). In addition to the guanine nucleotide binding domains, the presumptive effector domain is strictly conserved in the proteins encoded by the *ras* and *rap* genes and it has been suggested that they interact with the same effector molecule to regulate a common signal transduction pathway (3). Consistent with this suggestion, is the recent demonstration that overexpression of the human *rap*1A gene (termed K*rev*-1) suppressed the malignant phenotype associated with *ras* transformed NIH3T3 cells (7). The mechanism of this suppression is unknown but the rap protein may compete for a regulatory molecule and thereby modulate the activated ras protein.

It has been suggested that ras proteins have a function in both growth and differentiation but their precise role is not understood (8). The cellular slime mold *Dictyostelium discoideum* has been shown to contain at least two *ras* genes (9,10) and is an attractive organism for studies on *ras* gene function, since cellular proliferation and cellular differentiation are mutually exclusive processes. In the presence of a bacterial food source amoebae proliferate, but upon starvation cell division ceases and a relatively simple developmental process is initiated. Amoebae are attracted to one another by pulses of cyclic AMP to form a multicellular aggregate and then differentiate into one of two cell types, spore cells and stalk cells, that make up the terminal mature fruiting body. The two known *ras* genes, Dd*ras* and Dd*ras*G, are expressed at different developmental stages. Dd*ras* is maximally expressed during the pseudoplasmodial stage of development and is preferentially enriched in the prestalk cells (9), whereas Dd*ras*G is expressed during growth and early development (10). The combined expression of the two *ras* genes correlates with the observed rates of ras protein synthesis (11–13) although this does not preclude the possibility that the genome contains other *ras* related sequences. When the Dd*ras* or Dd*ras*G cDNAs were used to probe Southern blots of *Dictyostelium* genomic DNA under conditions of low stringency, they hybridized to both genes and also hybridized weakly to a number of other fragments. We proposed that these weakly hybridizing fragments represented other *ras* genes and/or *Dictyostelium* homologues of the *ras* gene superfamily (10). In this report we describe the characterization

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of a *Dictyostelium* gene that is highly conserved relative to the *rap* genes from human cells.

MATERIALS AND METHODS

Isolation and sequencing of the Dd*rap*1 clones

A number of clones were selected from a λgt10 cDNA library that hybridized to the Dd*ras*c1 probe under low but not high stringency conditions (10). DNA was isolated from liquid lysates (14) and digested with EcoRI. The resulting fragments were electrophoresed and transferred to nitrocellulose filters (15). The filters were prehybridized for 2−4 hrs at 37°C using a solution containing 30% formamide, 5×SSC (where 1×SSC is 0.15 M NaCl, 0.015 M sodium citrate); 5× Denhardt's (1×Denhardt's is 0.02% Ficoll, 0.02% bovine serum ablumin and 0.02% polyvinylpyrrolidone); 50 mM phosphate buffer pH 6.5; 0.5% SDS; 250 μg/ml salmon sperm DNA and hybridized for 16 hours at 37°C in a solution containing 30% formamide, 5×SCC; 1× Denhardt's; 20 mM phosphate buffer pH 6.5; 0.5% SDS; 100 μg/ml salmon sperm DNA and 10^6 cpm/ml of randomly primed Dd*ras*G-c3 insert (16). The filters were then washed under low stringency conditions (0.1×SSC, 0.1% SDS at 50°C) and exposed against X-ray film for 24 hr. Finally, the filters were washed under higher stringency conditions (0.1×SSC, 0.1% SDS at 65°C) and reexposed to X-ray film. The filters were then reprobed with Dd*ras*c1 and washed using an identical protocol. A single clone, c51, that hybridized to both Dd*ras*G-c3 and Dd*ras*c1 under low but not high stringency conditions was selected for further study. c51 was used to isolate five additional clones under high stringency conditions (0.1×SSC; 0.1% SDS at 65°C) from a λgt11 cDNA library, prepared from mRNA from cells at 3−4 hours of development. All cDNA probes were labelled using the random primer method (16).

The six cDNA clones were subcloned into M13 mp 18 (17) in both orientations and single stranded DNA from each was sequenced by the dideoxy chain termination method (18) using the modified T7 polymerase (United States Biochemical). The terminal 5′-sequence of the Dd*rap* cDNAs was obtained by sequencing complementary RNA from vegetative cells (10 μg) using AMV reverse transcriptase (19) and a synthetic oligonucleotide that was complementary to the 5′ end of the incomplete cDNA.

Isolation of DNA and RNA

The Ax-2 strain of *D. discoideum* was grown in rich nutrient media to stationary phase and then harvested by centrifugation at 700×g. The nuclei were isolated as described by Cocucci and Sussman (20) and the genomic DNA extracted (15).

D. discoideum strain V12-M2, was grown on nutrient agar plates in association with *Enterobacter aerogenes* (21). Differentiation was induced by separating the vegetative amoebae from the bacteria by four low speed centrifugations (700×g for 2 min) and plating 10^8 cells on Millipore filters resting on support pads saturated with phosphate buffer (21). Total cytoplasmic RNA from various stages of development was isolated (22) and subsequent isolation of polyadenylated RNA was performed by oligo d(T) cellulose chromatography (23).

Southern and Northern blot analyses

For Southern blots, genomic DNA was digested with various restriction enzymes (Bethesda Research Laboratories), size fractionated on agarose gels and then transferred to nitrocellulose. The filters were prehybridized and hybridized using the conditions described under clone isolation. The filters were washed two times (30 min per wash) in 2×SSC, 0.1% SDS at 50°C to provide low stringency conditions. After the filters were exposed to X-ray film, they were washed twice in 0.1 SSC, 0.1% SDS at 65°C to provide high stringency conditions and were then reexposed to X-ray film.

RNA samples for Northern blot analysis were adjusted to 50% formamide, 40 mM 3-(N-Morpholino) propanesulfonic acid pH 7.0, 10 mM sodium acetate, 1 mM EDTA and 6% formaldehyde, heat denatured for 10 min at 65°C and then size fractionated on 1.5% formaldehyde-agarose gels (24). The gels were partially hydrolyzed (15) and then transferred to nitrocellulose. The filters were prehybridized and hybridized as for the Southern blots (10), except that the buffers also contained 30 μg/ml polyadenylic acid. The filters were then washed using the same conditions as for the high stringency Southern blots.

RESULTS AND DISCUSSION

A clone that hybridized to both the Dd*ras* and Dd*ras*G cDNAs under low stringency conditions but failed to hybridize to either under higher stringency conditions was selected. This clone was used to select five additional clones under high stringency

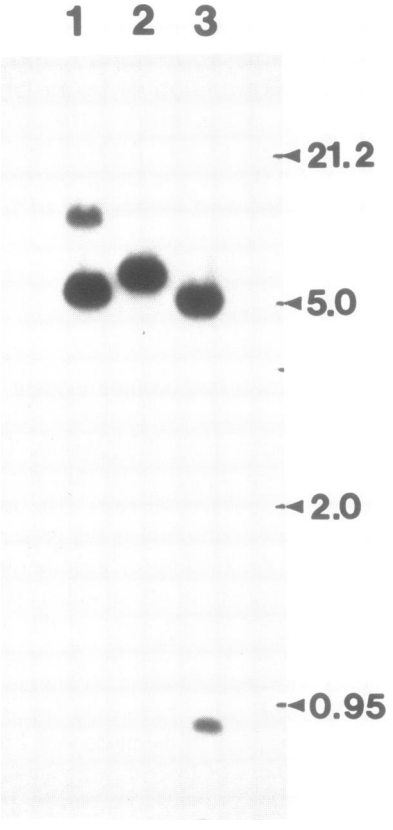

Figure 1. Southern blot analysis of the *Dictyostelium* Dd*rap*1 gene. Genomic DNA (5μg) from the *Dictyostelium discoideum* strain Ax-2 was digested with BglII (lane 1), EcoRI (lane 2) and EcoRI/BglII (lane 3), electrophoresed on a 0.8% agarose gel and then transferred to nitrocellulose. The filter was hybridized with Dd*rap*1 cDNA using previously described hybridization conditions (10). The filter was then washed two times (20 mins each) in 0.1 SSC, 0.1% SDS at 65°C.

conditions from a λgt11 cDNA library. When the isolated cDNA clones were used to probe a Southern blot of *Dictyostelium* genomic DNA they all hybridized to the same genomic fragment (Fig. 1) which corresponded to one of the weakly hybridizing fragments that had been previously observed using the Dd*rasG* and Dd*ras* cDNA probes under low stringency conditions (10). Two genomic fragments hybridized to the cDNA clone when digested with BglII or with EcoRI/BglII (Figure 2 lanes 1 and 3) because there is an internal BglII restriction site.

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1   GAATATATATTATAAACCAGATGCCTCTTAGAGAATTCAAAATCGTCGTTTTAGGTTCAG
                  M  P  L  R  E  F  K  I  V  V  L  G  S  G   14
61  GTGGTGTAGGTAAATCTGCTTTGACTGTGCAATTTGTTCAAGGTATTTTTGTTGAAAAGT
    G  V  G  K  S  A  L  T  V  Q  F  V  Q  G  I  F  V  E  K  Y   34
121 ACGATCCAACCATCGAAGATTCCTACAGAAAACAAGTCGAAGTTGATAGCAATCAATGCA
    D  P  T  I  E  D  S  Y  R  K  Q  V  E  V  D  S  N  Q  C  M   54
181 TGTTAGAAATTTTAGATACAGCTGGTACTGAACAATTTACTGCAATGAGAGATCTTTACA
    L  E  I  L  D  T  A  G  T  E  Q  F  T  A  M  R  D  L  Y  M   74
241 TGAAAAATGGTCAAGGTTTTGTTTTAGTATATTCAATCATTTCAAACTCCACTTTTAACG
    K  N  G  Q  G  F  V  L  V  Y  S  I  I  S  N  S  T  F  N  E   94
301 AGTTACCAGATCTCCGTGAACAAATTCTCAGAGTTAAGGATTGTGAAGATGTTCCAATGG
    L  P  D  L  R  E  Q  I  L  R  V  K  D  C  E  D  V  P  M  V  114
361 TTCTTGTTGGTAACAAATGCGATCTCCACGACCAACGTGTTATTAGCACAGAACAAGGTG
    L  V  G  N  K  C  D  L  H  D  Q  R  V  I  S  T  E  Q  G  E  134
421 AAGAACTCGCTCGTAAATTTGGTGATTGTTACTTTTAGAAGCATCTGCCAAGAATAAAG
    E  L  A  R  K  F  G  D  C  Y  F  L  E  A  S  A  K  N  K  V  154
481 TTAATGTTGAACAAATTTTCTATAACTTAATCCGTCAAATCAACCGTAAAAACCCAGTTG
    N  V  E  Q  I  F  Y  N  L  I  R  Q  I  N  R  K  N  P  V  G  174
541 GTCCACCAAGCAAAGCTAAATCAAAATGTGCTTTATTGTAAACAATCCATCAACTCTCCA
    P  P  S  K  A  K  S  K  C  A  L  L                          187
601 ACACCCTTCCATACTCACCCACCCATTTCAAATGTAACAATTGAAAAACAGAAAAAAAAA
661 AAAAAAAAAACAGAAAAAAAAAA
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Figure 2. Nucleotide and derived amino acid sequences of the Dd*rap*1 gene. The synthetic oligonucleotide primer that was used to complete the 5′ sequence is complementary to nucleotides 107 to 89. The numbers on the left indicate nucleotide positions in the sequence and the numbers to the right indicate the amino acid positions.

Sequence analysis confirmed that all six cDNA clones were derived from the same mRNA and thus represented the same gene. The cDNA clones did not contain the initiating methionine codon and all terminated within 12 nucleotides of each other, suggesting that the mRNA contained a particular sequence that caused the reverse transcriptase to terminate during the cDNA synthesis. The remaining 5′ sequence was obtained by sequencing vegetative RNA with a synthetic oligonucleotide that was complementary to the 5′ end of the incomplete cDNAs. The complete nucleotide sequence and the amino acid sequence deduced from the single open reading frame are shown in Fig. 2. The predicted amino acid sequence contains the four guanine nucleotide binding domains that are characteristic of the ras family of proteins, but there are considerable differences relative to the *Dictyostelium* ras proteins (Fig. 3). Ddras and DdrasG proteins are only 52% and 55% identical to the predicted sequence, whereas the human rap1A protein is 76% identical (Fig. 3). These results suggest that the protein is a human rap protein homologue and we have therefore given the gene the designation Dd*rap*1. The predicted Ddrap1 protein like its human homologue contains a threonine residue instead of the more characteristic glutamine in one of the conserved guanine nucleotide binding domains. Despite an otherwise high level of conservation within its N-terminal portion, the *Dictyostelium* protein differs from the human rap proteins at the extreme amino terminus in that it is two amino

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                                                                  31
Ddrap1   M P L R E F K I V V L G S G G V G K S A L T V Q F V Q G I F V E K
rap1A        M - - Y - L - - - - - - - - - - - - - - - - - - - - - - - -
DdrasG       M T - Y - L - I V - G - - - - - - - - - I - L I - N H - I D E
Ddras        M T - Y - L - I V - G - - - - - - - - - I - L I - N H - I D E

                                                              *   64
Ddrap1   Y D P T I E D S Y R K Q V E V D S N Q C M L E I L D T A G T E Q F
rap1A    - - - - - - - - - - - - - - - - C Q - - - - - - - - - - - - - - -
DdrasG   - - - - - - - - - - - - - - - T I - E E T - L - D - - - - - Q - E Y
Ddras    - - - - - - - - - - - - - - - S I - D E T - L - D - - - - - Q - E Y

                                                                  97
Ddrap1   T A M R D L Y M K N G Q G F V L V Y S I I S N S T F N E L P D L R
rap1A    - - - - - - - - - - - - - - - - A - - - - - T A Q - - - - D - Q - - -
DdrasG   S - - - - Q - - R T - - - - L C - - - - T - R - S - D - I A S F -
Ddras    S - - - - Q - - R T - - - - L C - - - - T - R - S Y D - I A S F -

                                                                 130
Ddrap1   E Q I L R V K D C E D V P M V L V G N K C D L H D Q R V I S T E Q
rap1A    - - - - - - - - T - - - - - - I - - - - - - - - - E - E - - V G K - -
DdrasG   - - - - - - - - K D R - - - I V - - - - - - - E S D - Q V T - G E
Ddras    - - - - - - - - K D R - - L I - - - - - A - - D H E - Q V - V N E

                                                                 163
Ddrap1    G E E L A R K F G D C Y F L E A S A K N K V N V E Q I F Y N L I R
rap1A     - Q N - - - Q W C N - A - - - S - - - S - I - - N E - - - D - V -
DdrasG    - Q D - - K S - - S P - - - - T - - - I R - - - - E A - - S - V -
Ddras     - Q - - - K D S L S - - H - S - - - S R I - - - E A - - S - V -

Ddrap1   Q I N R K N P V G P P S K A K S K           C A L L
rap1A    - - - - - T - - E K K K P K - K S           - L - -
DdrasG   E - R K D L K G D S K P E K G K - K R P L K A - T - -
Ddras    E - R K E L K G D Q S - G K A Q - K K K Q     - L I -
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Figure 3. Comparison of the derived amino acid sequence of the *Dictyostelium rap* protein (Dd*rap*1) with the sequences of Dd*ras* (8), Dd*rasG* (10) and the human *rap*1A proteins (3). The sequences are aligned relative to Dd*rap*1 but the numbers correspond to the amino acid positions in the human *rap* sequence. A dash indicates identity with Dd*rap*1 and only amino acids that differ are indicated by the single letter amino acid code. Gaps have been inserted to maximize the identity between the sequences. The four guanine nucleotide binding domains that are characteristic for the *ras* gene superfamily (1,2) have been boxed and the variant threonine at position 61 is marked by an asterisk.

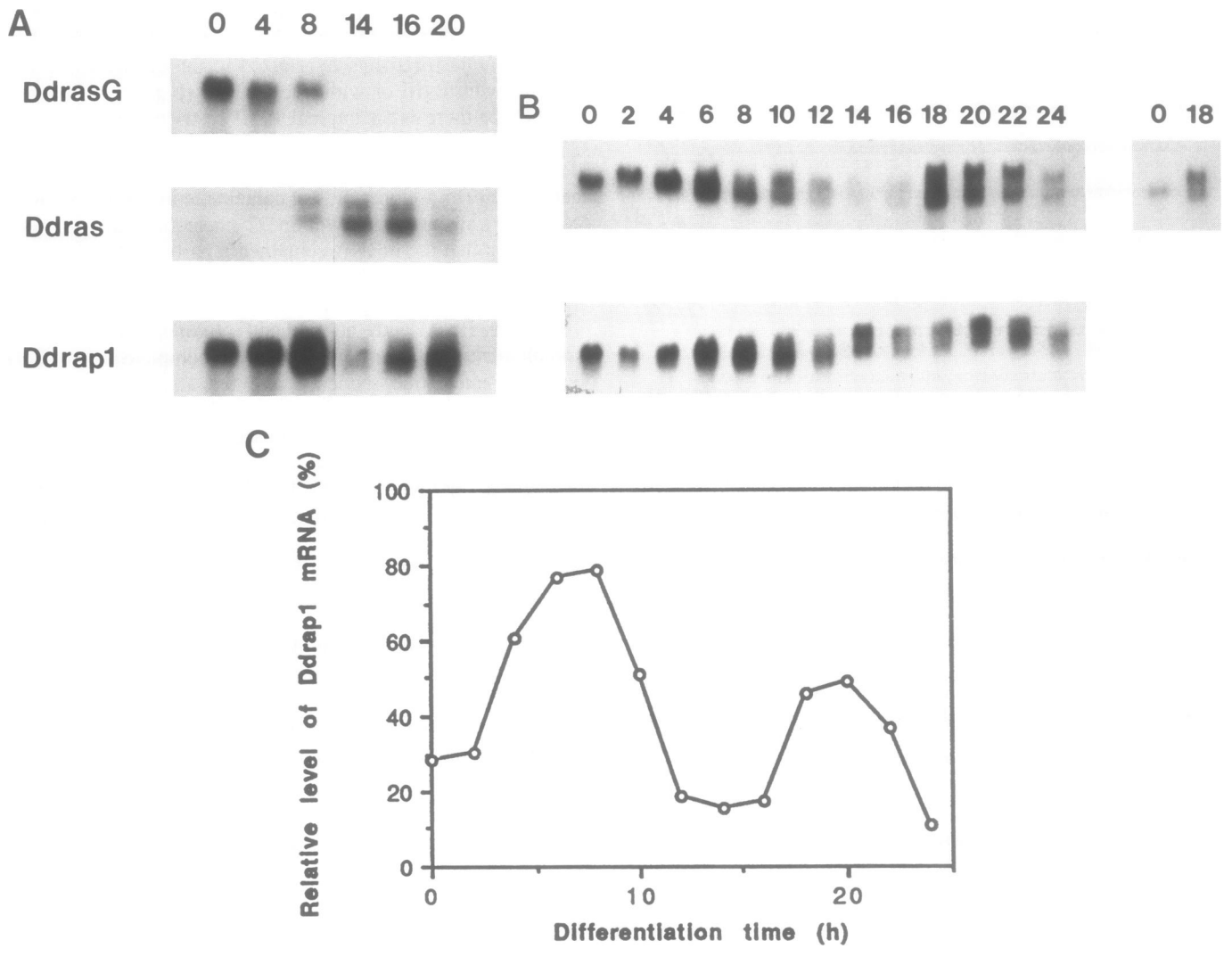

Figure 4. Expression of Dd*rap*1 during *Dictyostelium* development. At the indicated times (hours) after the onset of differentiation total RNA was extracted as and processed as described under Methods. A. Polyadenylated RNA (5μg) was probed with Dd*ras*G, Dd*ras* and Dd*rap*1 cDNAs as indicated. B. Total RNA (20 μg) from two independent experiments was probed using Dd*rap*1 cDNA. 0 hour and 18 hour samples were electrophoresed side by side to allow direct size comparison. The single 0 hour mRNA species was of 1.1 kb, the two 18 hour mRNA species were 1.0 and 1.3 kb. C. The amounts of Dd*rap*1 mRNA indicated in the three experiments were quantified by densitometry. For each experiment, expression was normalized relative to the maximum expressed value (100%). The data points represent the averages of the normalized values for the three experiments.

acids longer and three of the next six amino acids are different (Fig. 3). The phylogenetic conservation of the rap protein between humans and the lower eukaryote, *Dictyostelium*, is even higher than the level of conservation between the *ras* proteins. This high level of amino acid identity across such a wide range of species, suggests that the gene product fulfills an essential function.

In order to determine when the Dd*rap*1 gene was expressed, Northern blots of RNA isolated from various times during *Dictyostelium* development were hybridized with one of the Dd*rap*1 cDNA clones. A complex pattern of Dd*rap*1 specific messages was observed (Fig. 4). The amount of mRNA that hybridized to the Dd*rap*1 cDNA changed dramatically during development reaching maximum levels at the aggregation stage (6−8 hrs) and during culmination (18−22 hrs). During vegetative growth and early development a single 1.1 kb transcript was observed, but by 6−8 hours of development this transcript was no longer detectable and two new transcripts of 1.0 and 1.3 kb were apparent. There was no enrichment of the 1.0 or 1.3 kb Dd*rap*1 mRNAs in either prestalk or prespore cells (data not shown). All three mRNAs appeared to be derived from the Dd*rap*1 gene since they hybridized under high stringency conditions. In addition when the synthetic oligonucleotide complementary to the 5′ end of the cDNAs was used to sequence RNA from the pseudoplasmodial stage, the sequence obtained was identical to that for vegetative RNA. These results suggest that the 1.0, 1.1 and 1.3 kb transcripts are derived by alternate splicing or modification of the 3′ terminus of the Dd*rap*1 mRNA, although we can not totally rule out the possibility that the Northern hybridizations represent the sum of expression of more than one highly related Dd*rap* gene. Low stringency Southern blots with the Dd*rap*1 cDNA revealed an additional band which may represent a second *rap* gene or an additional member of the *ras* gene superfamily (data not shown). When the Northern blots shown in Fig. 3 were reprobed with Dd*rap*-c51 at lower stringency additional mRNA species were not revealed.

When the developmental expression of the Dd*rap*1 gene is compared to that of the two *Dictyostelium ras* genes, Dd*ras*G and Dd*ras* an interesting relationship is apparent (Fig. 4). Dd*ras*G

is maximally expressed between 0 and 4 hours of development, whereas Dd*ras* is maximally expressed between 12 and 16 hours (Fig. 4 and unpublished observations). The maximum levels of the Dd*rap*1 specific mRNA are, therefore, expressed during *Dictyostelium* development at stages where the levels of Dd*ras*G and Dd*ras* mRNA are declining. Although we have no direct evidence as to the function of Dd*rap*1 in *Dictyostelium*, the reciprocal nature of Dd*rap*1 gene expression with respect to the two *ras* genes suggests the possibility that the *ras* and *rap* gene products in *Dictyostelium* have antagonistic regulatory roles. Furthermore, the biphasic pattern of Dd*rap*1 expression suggests the possibility that the Ddrap1 protein antagonizes both the Dd*ras*G and Dd*ras* gene products. An antagonistic interaction between *ıras* and *rap* gene products has already been suggested for human cells (7).

The precise physiological function of the *ras* gene products in *Dictyostelium* is not known, but it has been shown that transformation of *Dictyostelium* with activated Dd*ras* (substitution of threonine for glycine at amino acid 12) results in the formation of pseudoplasmodia with multiple tips during the differentiation process (25). Evidence has been presented to suggest that this phenotype is due to an impairment of signal transduction involving the cyclic AMP cell surface receptor and altered levels of inositol triphosphate (26–28). Since the overexpression of the human *rap* gene suppresses the transformed phenotype associated with an activated *ras* gene in NIH3T3 cells, it will be interesting to determine if the mutant phenotype of the activated Dd*ras* transformant will be suppressed by overexpression of the *Dictyostelium* Dd*rap*1 gene.

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