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cDNA and derived amino acid sequence of the hypusine containing protein from *Dictyostelium discoideum*

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The eukaryotic translation initiation factor eIF-4D is the only protein known to contain the unusual amino acid hypusine, a posttranslationally modified lysine. For the production of monoclonal antibodies the hypusine-containing protein (HP) was isolated from *Dictyostelium discoideum*. Using these monoclonal antibodies, a full-length cDNA clone was isolated from a λgt11 library. The *D. discoideum* HP consists of 169 amino acids and has a molecular mass of 18.3 kDa. It is encoded by a single gene. Tryptic and cyanogen bromide peptides were prepared from the purified protein and sequenced. The hypusine residue is located at amino acid position 65 of the HP. The corresponding mRNA of approx. 0.6 kb is present throughout the life cycle of *D. discoideum*.

Initiation factor eIF-4D; Posttranslational modification; Polyamine; Hypusine

1. INTRODUCTION

The putative eukaryotic translation initiation factor eIF-4D is the only cellular protein known so far to contain the unusual amino acid hypusine [*N*-(4-amino-2-hydroxybutyl)lysine] [1,2]. Peptide-bonded hypusine is formed by a two-step post-translational modification: a specific lysine residue of the protein is conjugated with the 4-aminobutyl moiety of the polyamine spermidine to form the intermediate deoxyhypusine which is subsequently hydroxylated to form hypusine [3]. The lysine residue is modified immediately after or even during the synthesis of the protein and the hypusine is not altered thereafter [4,5]. The protein remains stable up to 72 h after its synthesis [6,7]. In cell-free systems eIF-4D has been shown to enhance the formation of methionyl-puromycin [8], and to promote translation of polyuridylic acid to form polyphenylalanine [9]. Furthermore, eIF-4D stabilizes all intermediate complexes made during 80 S initiation complex formation [10]. However, the factor has been shown not to influence translation of native globin mRNA under cell-free conditions [9] except for a slight downward shift of the optimum magnesium concentration for globin synthesis in the absence of spermine [11]. Its role as an initiation factor remains therefore to be established.

Considering that the hypusine-containing protein (HP) and the enzyme systems responsible for the modification of this specific lysine residue are conserved among such divergent eukaryotes as sea urchin [12], yeast *Drosophila* [13], and mammalians [14–18], HP is likely to play an important role within cells. Up to now, hypusine has not been detected in prokaryotes such as *Escherichia coli* and *Methanococcus voltae* [13].

In order to investigate further the function of HP, especially the role of the hypusine residue, the protein was isolated from *Dictyostelium discoideum* a lower eukaryote whose amoeboid cells aggregate upon starvation and finally form a multicellular fruiting body. The study of the protein function is facilitated by transforming cells with integrating or extrachromosomally replicating vectors [19–21]. Genes can be inactivated by homologous recombination with a gene fragment carried by a transformation vector [22–24].

In this paper we report the isolation and purifica-

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tion of HP from *D. discoideum*, preparation of monoclonal antibodies, amino acid sequence analysis of tryptic and cyanogen bromide peptides, isolation and sequence of a full-length cDNA encoding HP.

2. MATERIALS AND METHODS

2.1. *Growth and development of* D. discoideum

D. discoideum strains AX2 and HG220 [25] were grown axenically at 21°C [26,27] for protein and nucleic acid preparation. For the study of transcriptional and translational regulation during development, AX2 cells were grown up to a density of 2–5 × 10^6 cells/ml, washed with 17 mM Soerensen phosphate buffer, pH 6.0, and deposited on Millipore filters [28]. The filters were incubated at 21°C, and cells were harvested at different stages of development for RNA isolation. For the assay of in vivo translation, AX2 cells were grown and washed as described above, pelleted for 5 min at 1500 rpm and resuspended in phosphate buffer. After adjusting the cell density to 1 × 10^7 cells/ml, the cells were further incubated at 21°C with 5 μCi/ml [^{3}H]spermidine (24.3 Ci/ml, Amersham, Braunschweig) and samples were taken every 4 h.

2.2. *HP purification for monoclonal antibody production*

HP was isolated from *D. discoideum* strain HG220. 2.2 × 10^{10} washed cells were frozen at −20°C, the lysate was centrifuged (20000 × *g*, 30 min). All further steps were performed at 4°C. The supernatant was incubated for 2 h with DE52 cellulose (Whatman, Maidstone) containing 50 mM Tris-acetate, pH 6.8, 1 mM dithiothreitol, 0.1 mM EDTA [29]. The material was loaded onto a column (190 cm^3). The column was washed with buffer I (50 mM Tris-acetate, pH 6.8, 1 mM dithiothreitol, 0.1 mM EDTA), and subsequently with buffer I containing 100 mM potassium chloride. The HP was eluted with buffer I containing 350 mM potassium chloride (flow rate: 1.3 ml/min). HP was monitored by the determination of hypusine in ninhydrin amino acid analysis as described below. HP containing fractions were applied to a Sephacryl S 200 column (890 cm^3, Pharmacia, Freiburg) [29]. The HP was eluted with 150 mM ammonium acetate, pH 7.8, 1 mM dithiothreitol (flow rate, 0.35 ml/min) and further purified by reversed-phase high-performance liquid chromatography (HPLC) using a Vydac C4 column (12.5 cm × 4 mm^2, 5 μm, Science Service, München); buffer A: 0.1% trifluoroacetic acid in water and buffer B: 0.1% trifluoroacetic acid in acetonitrile. The gradient was 20–40% B in 20 min/40–45% B in 15 min/45–60% B in 10 min (flow rate, 0.5 ml/min). For the subsequent size exclusion chromatography the HP containing fraction was reduced to 100 μl (Speedvac, Savant) and applied to a TSK 2000 SW column (Pharmacia, Freiburg), and HP was eluted with 10% acetonitrile/0.1% trifluoroacetic acid.

2.3. *Amino acid analysis*

For amino acid analysis, 0.5 nmol samples of peptide material recovered from HPLC were subjected to gas-phase hydrolysis applying 200 μl of a mixture of trifluoroacetic acid/7.2 M HCl (1:10, v/v) at 159°C for 20 min. The hydrolysed mixture was analysed on a ninhydrin amino acid analyzer (Beckman 6300), using a buffer-system in which glucosamine, galactosamine and hypusine separate from other ninhydrin-reactive compounds.

2.4. *Production of monoclonal antibodies*

The purified protein was used for the production of monoclonal antibodies. Two female BALB/c mice were immunized with purified HP (125 μg HP per mouse) supplemented with either complete Freund's adjuvant (Behring, Frankfurt) or Alugel S (Serva, Heidelberg). Spleen cells were fused with myeloma cells Ag8653 or PAJ-B3Ag8I [30] using polyethylene glycol (M_r = 4000) (Polysciences, Warington). Hybridoma supernatants were tested using total cell lysate prepared from *D. discoideum* cells, separated on 12% polyacrylamide gels and blotted onto immobilon membranes (Millipore, Neu-Isenburg) essentially as described for siliconized glass fiber [31]. The blots were labeled with biotinylated anti-mouse antibody and streptavidin-biotinylated peroxidase both in a dilution of 1:400 (Amersham, Braunschweig) [32]. Positive clones were further subcloned in microtiter plates according to [33].

2.5. *Purification of HP by immunoaffinity chromatography*

Monoclonal antibodies were covalently coupled to a protein A-Sepharose C1-4B column (Pharmacia, Freiburg) according to [34]. After applying the soluble fraction from 4.5 × 10^{10} *D. discoideum* cells onto the column (1.7 cm^3), the column was washed extensively with 140 mM sodium phosphate, pH 8.0. HP was eluted with 50 mM sodium phosphate, pH 11 [35]. The eluted fractions were immediately rechromatographed by reversed-phase HPLC on a Zorbax protein-plus column (DuPont, Bad Homburg), using a 0.1% trifluoroacetic acid/water (A) and 0.1% trifluoroacetic acid/acetonitrile (B) solvent system (flow rate, 1.0 ml/min). A linear gradient from 20 to 50% B in A was applied over a period of 50 min at room temperature. Peaks were collected manually.

2.6. *Amino acid sequence analysis*

Tryptic and cyanogen bromide peptides were prepared and isolated essentially as described in [36]. The cleavage of methionyl bonds was performed with 10% cyanogen bromide (w/v) in 70% (v/v) formic acid at room temperature for 2 h in the dark. The tryptic cleavage was carried out using a weight ratio of trypsin (Worthington, Freehold) to substrate of 1:50. The reaction was performed in 100 mM ammonium hydrogen carbonate, 10 mM calcium chloride, pH 7.0, for 8 h at 37°C. Proteolysis was stopped by acidifying the solution with formic acid. The resulting cleavage mixture was separated by reversed-phase HPLC. The sequence of the purified peptides was determined on a gas-phase sequencer 470 A (Applied Biosystems, Foster City). The phenylthiohydantoin amino acids were separated using an isocratic HPLC system [37].

2.7. *Isolation of cDNA clones*

A gt11 cDNA expression library, kindly provided by Drs Kessin and Lacombe, Columbia University, was screened with a mixture of all monoclonal antibodies obtained using biotinylated anti-mouse antibody and streptavidin-biotinylated peroxidase both in a dilution of 1:200 (Amersham, Braunschweig). The cDNA library was prepared from AX3 *D. discoideum* cells that had been induced by cAMP [38]. Recombinant phages were multiplied on *E. coli* RY1088 for DNA isolation. The *Eco*RI (Boehringer, Mannheim) digested DNA was

separated in a 0.8% agarose gel in Tris-acetate buffer, pH 8.0 (TAE) [39]. The largest cDNA insert was isolated by electroelution [39] and recloned into bluescript vector (Stratagene, Heidelberg) using the *E. coli* strain DH5α as host. For sequencing of both strands, the insert was further subcloned using the unique *Eco*RV restriction site at position 268 (fig.1).

2.8. *DNA sequencing*

Sequencing of both strands was performed by the dideoxynucleotide chain termination method [40] using the sequenase sequencing kit (United States Biochemical, Cleveland) and universal pUC/M13 uni and reverse primer (Boehringer, Mannheim). The sequence was analysed with programs from the University of Wisconsin, Genetic Computer Group, UWGCG.

2.9. *DNA and RNA isolation from* D. discoideum *and hybridization analysis*

Chromosomal DNA was isolated from partially purified nuclei. 5×10^9 cells were lysed with 100 ml Nonidet P-40 buffer (10 mM magnesium acetate, 10 mM sodium chloride, 30 mM Hepes, pH 7.5, 10% (w/v) sucrose, 2% (w/v) Nonidet P-40), and the nuclei pelleted by a low-speed spin. The nuclei were lysed at 60°C with EDTA-sarcosyl (2% sarcosyl (w/v), 200 mM EDTA, pH 8.4), and the lysate subjected to cesium chloride-ethidium bromide density-gradient centrifugation (0.92 g cesium chloride per g solution) [41]. DNA digested with restriction endonucleases was separated on 1% agarose gels in Tris-borate buffer (TBE) [39], transferred to nitrocellulose filters (BA85, Schleicher & Schuell, Dassel) and probed with

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1                                  5' acaggtcatattttaattaataccaataataattgca    37

38  atg aaa cca tta ata atg gag tac aac aaa atg tca gat aac gaa                82
1   M   K   P   L   I   M   E   Y   N   K   M   S   D   N   E                 15
    HindIII
83  gct tta gat gtc gaa gac gcc ggt tca ggt gct tca ggt gct tca               127
16  A   L   D   V   E   D   Y   A   Q   A   G   S   G   A   S                 30

128 tta acc ttc cca att caa tgt tca gca tta aga aag aac ggt ttc               172
31  L   T   F   P   I   Q   C   S   A   L   R   K   N   G   F                 45

178 gtc gtc att aaa ggt ttc cca tgt aag att gtt gat atg tca act               217
46  V   V   I   K   G   F   P   C   K   I   V   D   M   S   T                 60

218 tcc aaa acc ggt aaa cac ggt cac gcc aaa gtt aac atc act gct               262
61  S   K   T   G   K*  H   G   H   A   K   V   N#  I   T   A                 75
        EcoRV
263 atc gat atc ttc act ggt aag aaa tac gaa gaa att tgc cca tca               307
76  I   D   I   F   T   G   K   K   Y   E   E   I   C   P   S                 90

308 act cac aac att gat gta cca aat gtc agc aga aag gaa tac acc               352
91  T   H   N   I   D   V   P   N#  V   S   R   K   E   Y   T                 105

353 gtt atg gat gtt caa gat ggt tac tta tca ctc ttg gat gct ggt               397
106 V   M   D   V   Q   D   G   Y   L   S   L   L   D   A   G                 120
                    Sau3A
398 ggt gaa gtc aaa gaa gat ctt gcc ctc cca gaa gat gat att ggt               442
121 G   E   V   K   E   D   L   A   L   P   E   D   D   I   G                 135

443 aaa gaa att acc caa atg tta aaa gaa ggt aaa gag cca tta gtt               487
136 K   E   I   T   Q   M   L   K   E   G   K   E   P   L   V                 150

488 tca gtc atc tct gct tta ggt aaa gaa ggt gtc gtc tct gtt aaa               532
151 S   V   I   S   A   L   G   K   E   G   V   V   S   V   K                 165

533 gtc agc aac aat taa attgtttaaaattctctttataaaaaa 3 '                      574
166 V   S   N   N
```

Fig.1. cDNA and derived protein sequence of the hypusine containing protein from *D. discoideum*. The sequence of both DNA strands was determined by the dideoxy chain termination method. The amino acid sequence of the eight tryptic and cyanogen bromide peptides determined by Edman degradation are underlined. The start (AUG, position 38) and stop codon (TAA, position 454) are double underlined. The restriction sites of *Eco*RV, *Sau*3A and *Hin*dIII are indicated with bold letters. Potential *N*-glycosylation sites at position 72 and 98 are marked with #. The position of the hypusine residue, a posttranslationally modified lysine residue, at position 65 of the protein is indicated as K*.

nick-translated cDNA insert in 50% formamide, 2 × SSC, 1% sarcosyl, 4 mM EDTA, 0.1% SDS, 4 × Denhardt's solution and 120 mM phosphate buffer, pH 6.8, at 37°C for 16–18 h [41]. The filters were washed for 1 h at 37°C using conditions present during hybridization.

Total cellular RNA was prepared after lysis of the cells with SDS (0.5% final concentration) and purified with four phenol/chloroform extractions [41]. For Northern blot analysis RNA was separated in 1.2% agarose gels in the presence of 6% formaldehyde. Hybridization was performed as described above.

3. RESULTS AND DISCUSSION

3.1. *Purification of HP and preparation of monoclonal antibodies*

For the induction of antibodies, HP was purified using a combination of different chromatographic separation methods as described in section 2. From 1×10^{10} cells, 340 μg purified HP was obtained. Two Balb/c mice were immunized with purified HP and spleen cells fused with myeloma cells after 8 weeks. Nearly all obtained hybridoma clones were positive. 35 hybridoma producing monospecific antibodies were isolated. Using a mixture of these monoclonal antibodies, a simple and rapid two-step purification method for HP was developed, based on immunoaffinity chromatography and subsequent reversed-phase HPLC. The HP obtained was pure by the criteria of 2D gel electrophoresis (not shown) and amino acid analysis which showed 1 mol of hypusine/mol of protein. Direct amino acid sequence analysis revealed that HP is N-terminally blocked.

3.2. *A cDNA clone coding for HP and its verification by DNA and protein sequences*

A cDNA library in λgt11 was screened using a mixture of all monospecific monoclonal antibodies against HP. Out of 1×10^5 pfu approx. 2000 positive clones were obtained, and 12 of them further purified. After *Eco*RI digestion, the largest cDNA insert of approx. 600 nucleotides was isolated, recloned into the bluescript vector, and its sequence determined. The cDNA insert consisted of 574 nucleotides and contained one open reading frame starting at position 38 with AUG and ending at position 545 with TAA (fig.1). TAA is the most often used stop codon in *D. discoideum*. The cDNA clone, although containing the entire coding region, is probably not complete. It seems to lack a few nucleotides of the 5′-untranslated region and part of the 3′-flanking region including the polyadenylation site. The open reading frame codes for 169 amino acids. The molecular mass of the putative protein calculated from the sequence is 18.3 kDa, which is close to the apparent molecular mass of about 21 kDa as determined by SDS-polyacrylamide electrophoresis (fig.4). The codon usage is comparable to that of other genes of *D. discoideum* which prefer AT-rich codons [42]. To confirm that the obtained cDNA clone encodes HP, several peptides of the HP protein purified from *D. discoideum* cells were sequenced. Since the amino terminus of the protein is blocked, the purified protein was cleaved with trypsin and cyanogen bromide. The peptides were isolated using reversed-phase HPLC and eight of them were

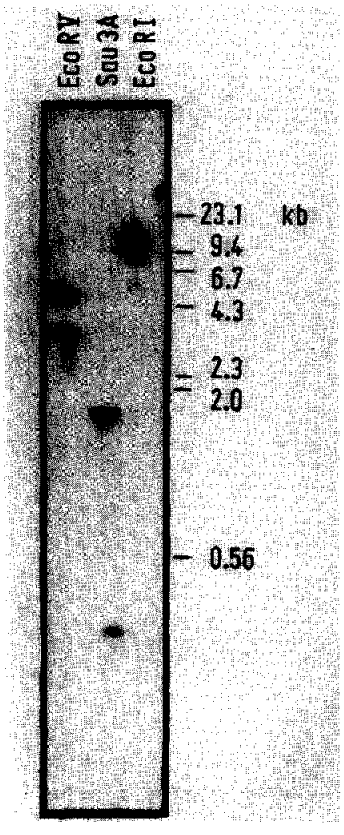

Fig.2. Identification of the HP gene as a single-copy gene. 5 μg of *Eco*RI, *Eco*RV and *Sau*3A-digested genomic DNA were separated on a 1% agarose gel and blotted onto a nitrocellulose filter. The filter was hybridized with 100 ng [^{32}P]dATP nick-translated cDNA insert of 0.57 kb. Sizes of restriction fragments of *Hin*dIII-digested DNA are indicated at the right.

sequenced by Edman degradation. The amino acid sequence of all eight peptides, corresponding to a total of 75 amino acid residues, could be recognized within the open reading frame. One tryptic peptide contained the hypusine residue which is located at position 65 of the cDNA-derived amino acid sequence of the protein. The sequence of this tryptic peptide derived from *D. discoideum* HP is identical with a peptide derived from eIF4D of human red blood cells [29], indicating that the sequence around the hypusine residue is highly conserved among such divergent species as *D. discoideum* and man. Furthermore, it could be verified that the HP contains no tryptophan [43]. Two potential *N*-glycosylation sites are located at position 72 and 98. However, the absence of amino sugars in the ninhydrin amino acid analysis suggests that HP is not glycosylated. Computer-based search on the nucleotide and protein level revealed no homology to any other known sequences.

3.3. *Genomic structure of the HP gene*

In order to investigate the genomic structure of the gene encoding HP, chromosomal DNA of *D. discoideum* strain AX2 was isolated and digested with *Eco*RI, *Eco*RV and *Sau*3A. Hybridization analysis using the ^{32}P-dATP labeled cDNA fragment of 0.57 kb revealed that the gene is a single-copy gene (fig.2). Digestion with the restriction enzyme *Eco*RI revealed a single band of approx. 11 kb. Digestion with *Eco*RV resulted in two bands of 5.5 and 3.4 kb and digestion with *Sau*3A in two bands of 1.8 and 0.44 kb. Both enzymes have a single restriction site within the cDNA insert (fig.1).

3.4. *Presence of HP mRNA and of the HP protein during all stages of development*

D. discoideum undergoes a developmental cycle in which upon starvation, the amoebae aggregate

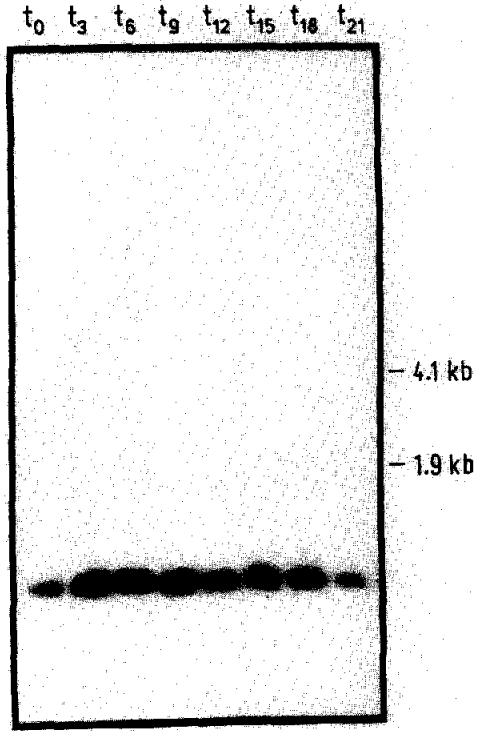

Fig.3. Expression of HP specific mRNA during development of *D. discoideum* strain AX2. 10 μg total RNA of each developmental stage (t_0–t_{21}) were separated on a 1.2% agarose gel in the presence of 6% formaldehyde, transferred onto a nitrocellulose filter and hybridized with 100 ng [^{32}P]dATP nick-translated cDNA insert of 0.57 kb. The sizes of the ribosomal RNAs are indicated at the right.

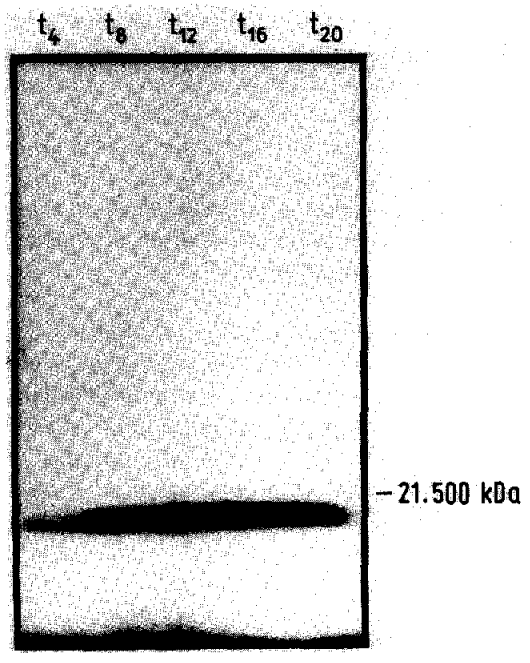

Fig.4. Incorporation of [^{3}H]spermidine into HP protein in aggregating AX2 cells. Starving AX2 cells (t_0) were incubated with 5 μCi/ml of [^{3}H]spermidine. At different time points aliquots of 1.5×10^7 cells were harvested, the cellular proteins separated on a 12% SDS-polyacrylamide gel and autoradiographed over 25 days. The position of trypsin inhibitor (21.5 kDa) is indicated on the right.

and form fruiting bodies. *D. discoideum* strain AX2 was harvested at various times during development, mRNA was isolated and equal amounts per time point were separated on a denaturing gel. Hybridization with the [^{32}P]dATP labeled cDNA fragment of 0.57 kb revealed that the mRNA was present throughout development. The levels of HP-specific RNA increased during the early stages of aggregation (t_3) and decreased in late development (t_{18}–t_{21}) (fig.3). Therefore, the HP-specific RNA is modulated during development, as it is known from other *D. discoideum* genes [33], but it is not strictly developmentally regulated. The continuous presence of the HP-specific mRNA is reflected on the protein level (fig.4). In order to confirm that the HP protein is continuously synthesized during development, starving cells of *D. discoideum* strain AX2 were incubated with [^{3}H]spermidine (t_0) and harvested at different time points. Proteins of whole cells were separated on 12% polyacrylamide gel and autoradiographed. The increase in the amount of radioactivity incorporated into HP in the early stages of development (t_4–t_{12}), is presumably due to accumulation of HP because of its long half-life. These experiments indicate that HP is expressed constitutively and, therefore, seems to be required during all developmental stages of *D. discoideum*.

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