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Biochemistry

Identification of the hypusine-containing protein Hy^+ as translation initiation factor eIF-4D

(post-translational modification/cell growth regulation/regulation of protein synthesis)

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ABSTRACT **A single protein of M_r 17,000–19,000 and pI ≈5.1, found in all animal cells we have studied to date, undergoes post-translational modification in growing cells to form the unusual amino acid hypusine. Because of the association of this modification with the increasing rate of protein synthesis during lymphocyte growth stimulation, its subcellular distribution, and its widespread occurrence and structural conservation among animal cells, we considered the possibility that this protein might be a translation initiation factor. Purified rabbit reticulocyte factors (eukaryote initiation factors) eIF-4C and eIF-4D were chosen for study because of their M_r (17,000–19,000) and acidic pI. The hypusine-containing protein and purified eIF-4D showed identity of electrophoretic mobility in both isoelectric focusing and $NaDodSO_4$/polyacrylamide gel electrophoresis dimensions, while eIF-4C was clearly nonidentical. Purified eIF-4D contained approximately 1 mol of hypusine per mol of protein. Since only one protein has thus far been observed to contain hypusine, we conclude that eIF-4D is the hypusine-containing protein. On the basis of relative synthesis among lymphocyte proteins and detection by Coomassie blue staining, we also conclude that eIF-4D is a major cell protein. It is possible that the activity of this factor is modulated by post-translational hypusine formation, which may play a role in regulation of protein synthesis during lymphocyte growth stimulation.**

Hypusine [N^{ϵ}-(4-amino-2-hydroxybutyl)lysine] is an unusual amino acid (1) formed by the addition of a butylamino group, derived from spermidine, to the side chain of a lysine residue in a peptide chain followed by hydroxylation at carbon 2 of the added group (2, 3). Hypusine has thus far been shown to occur naturally as a post-translational modification in only one protein, which we have termed Hy^+ (M_r, 17,000–19,000; pI, ≈5.1), in growing lymphocytes and fibroblasts of human and animal origin (2–4). This protein is probably formed in all growing eukaryotic cells. In quiescent lymphocytes and serum-deprived fibroblasts, hypusine formation is minimal although the substrate protein, Hy^0, is continuously synthesized. On stimulation of lymphocytes with a mitogen, the conversion of Hy^0 to Hy^+ increases detectably within a few hours. The rate of hypusine formation increases in parallel with the increasing rate of protein synthesis characteristic of activated lymphocytes (4). Thus, the production of Hy^+ is one of the relatively early events characterizing the activation of quiescent lymphocytes.

Hy from human lymphocytes and Chinese hamster ovary fibroblasts showed identical electrophoretic mobility in both electrofocusing and $NaDodSO_4$/polyacrylamide gel electrophoresis dimensions (4), indicating a widely distributed protein with well-conserved structure. Our preliminary observations showed that the great bulk of Hy^+ was found free in the cytosol but that small amounts were found in ribosomal and cytoskeleton preparations. The relationship of Hy^+ formation to increasing protein synthesis, its subcellular distribution, and its ubiquity among growing animal cells led us to consider whether Hy^+ might be involved in control of protein synthesis, possibly as one of the known translation initiation factors.

Among the 10 initiation factors described and purified to date (for review, see refs. 5 and 6), three [eukaryote initiation factor (eIF)-1, -4C, -4D] have molecular weights in the same range as Hy^+, and two of these are described as acidic proteins (eIF-4C and -4D) but, of the two, only eIF-4D has a subcellular distribution like that of Hy^+ (7). All of the eukaryotic initiation factors have been prepared from rabbit reticulocytes and their activities have been defined (8–10), which permitted comparison of the properties of highly purified eIF-4C and eIF-4D with those of Hy^+. On the basis of two-dimensional electrophoretic mobility and the recovery of hypusine from hydrolysates of purified initiation factors, we identify Hy^+ with eIF-4D.

MATERIALS AND METHODS

Human peripheral lymphocytes were prepared from heparinized venous blood and incubated at 10^6 per ml in RPMI 1640 medium/10% autologous plasma supplemented with penicillin and streptomycin as described (11). Nylon column adsorption was omitted, and monocytes were depleted by three 1-hr cycles of adherence to plastic tissue culture vessels. Rabbit lymphocytes were prepared similarly.

For specific labeling of hypusine, lymphocytes were stimulated to grow by addition of phytohemagglutinin (2 μg/ml; Sigma; type IV) followed by incubation for 40 hr in the presence of either [*terminal methylenes*-^{3}H(*N*)] spermidine or [2,3-^{3}H]-putrescine (2 μCi/ml; New England Nuclear; 31 Ci/mmol and >15 Ci/mmol, respectively). For [^{3}H]leucine labeling of all newly synthesized proteins, lymphocytes were transferred at 10^7 per ml to RPMI 1640 lacking leucine supplemented with 5% dialyzed fetal bovine serum and [^{3}H]leucine (150 μCi/ml; Amersham; 130 Ci/mmol) and incubated for 4 hr.

Radiolabeled cells were harvested and analyzed by two-dimensional polyacrylamide gel electrophoresis as described (4).

Analysis of hypusine and arginine contents of acid hydrolysates of purified initiation factors was carried out as outlined (12) by an automated ion exchange procedure using a Dionex D-400 analyzer, a three-buffer elution system, and fluorometric detection.

Rabbit reticulocyte initiation factors eIF-4C and eIF-4D were purified as described (8). The factor preparations used in this study were identified on polyacrylamide gels by M_r and pI and

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Abbreviations: Hy^+, hypusine-containing protein; Hy^0, Hy^+ substrate protein; eIF, eukaryote initiation factor.

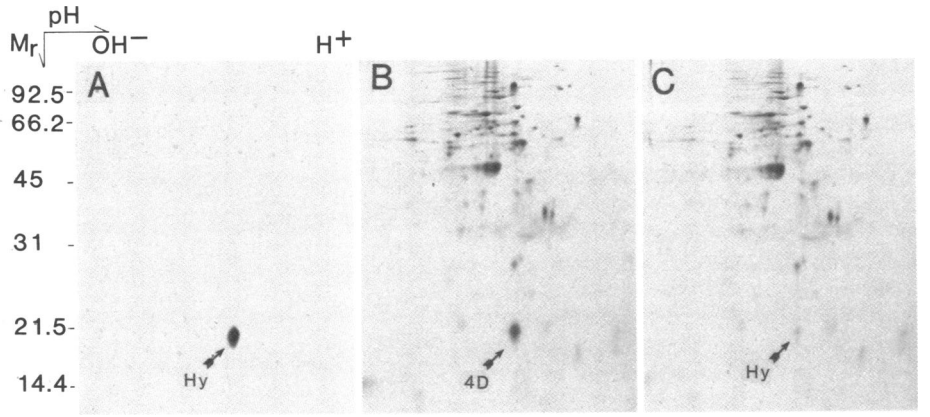

FIG. 1. Comparison of two-dimensional electrophoretic properties of human Hy^+ and rabbit reticulocyte eIF-4D. Human peripheral lymphocytes were incubated with phytohemagglutinin and [^{3}H]spermidine (2 μCi/ml) for 40 hr. Whole-cell proteins were analyzed by two-dimensional electrophoresis (4): first dimension, isoelectric focusing, pH 3.5–10 Ampholytes, 9,000 V·hr; second dimension, $NaDodSO_4$/12% polyacrylamide gel electrophoresis. (*A*) [^{3}H]Spermidine-labeled lymphocyte protein (100 μg) together with 2–4 μg of eIF-4D (unlabeled). A radiofluorograph is shown. (*B*) The gel shown in *A* was stained with Coomassie blue. (*C*) Lymphocyte protein (100 μg) without added eIF-4D. A Coomassie blue-stained gel is shown. Hy, Hy^+; 4D, eIF-4D. M_r markers are $\times 10^{-3}$.

by assay of methionylpuromycin synthesis directed by ApUpGp (9) and translation of globin mRNA (10). Methionylpuromycin was synthesized in 50 μl of 100 mM KCl/3 mM $MgCl_2$/20 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/0.4 mM puromycin, 0.5 mM GTP containing 0.3 A_{260} unit of ApUpGp, 20 pmol of 80S ribosomes, 20 pmol of [^{35}S]Met-tRNA$_i$ (50 Ci/mmol), 1.5 μg of eIF-2, 0.8 μg of eIF-5, 2 μg of eIF-4C, and 2 μg of eIF-4D. Globin mRNA was translated in 100 μl of 85 mM KCl/3 mM $MgCl_2$/20 mM Tris·HCl, pH 7.5/1 mM dithiothreitol/50 μM amino acid mixture including [^{14}C]valine (250 mCi/mmol)/1 mM ATP/0.2 mM GTP/4 mM phospho*enol*pyruvate containing 0.4 A_{260} unit of polysomes, 0.3 A_{260} unit of unfractionated reticulocyte tRNA, 50 μg of crude aminoacyl-tRNA synthetases, 4 units of pyruvate kinase, 1.5 μg of eIF-2, 2 μg of eIF-3, 8 μg of eIF-4A, 3 μg of eIF-4B, 1 μg of eIF-5, and 1 μg of eIF-4C.

RESULTS

Electrophoretic mobility in two dimensions of human lymphocyte Hy^+ was first compared with that of rabbit reticulocyte eIF-4C and eIF-4D, purified as described (8), and assayed for individual activities as described above. Rapidly growing human lymphocytes were radiolabeled with either [^{3}H]leucine or [^{3}H]spermidine, and whole cytoplasmic proteins were analyzed by two-dimensional electrophoresis with and without addition of 2–4 μg of purified unlabeled eIF-4C or eIF-4D. Comparison of stained gels permitted localization of the added initiation factor, and superimposition of radiofluorographs permitted determination of the correspondence of mobility between Hy^+ and initiation factors.

Hy^+ can be visualized on fluorographs of [^{3}H]spermidine-labeled preparations as the only significantly radiolabeled peptide (3, 4) (Fig. 1*A*). We have shown that all of the radioactivity in this peptide, derived from [^{3}H]spermidine, is recoverable as hypusine (3, 4). The stained peptide identified as eIF-4D in the same preparation (compare Fig. 1 *B* and *C*) was completely superimposable on the Hy^+ spot, demonstrating identity of the electrophoretic mobility. The heavy protein load used in the gels shown in Fig. 1 allowed visualization of Hy by staining in the control preparation, to which no eIF-4D had been added (Fig. 1*C*). This peptide was also superimposable on the eIF-4D spot (Fig. 1*B*).

Similar results were obtained when initiation factors were

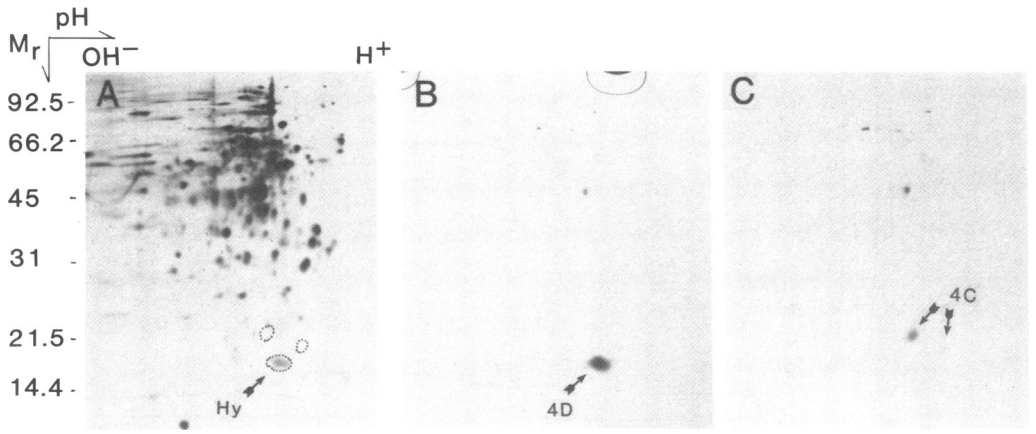

FIG. 2. Comparison of two-dimensional electrophoretic properties of [^{3}H]leucine-labeled human lymphocyte proteins, eIF-4D, and eIF-4C. Rapidly growing human peripheral lymphocytes were labeled with [^{3}H]leucine (150 μCi/ml) for 4 hr. Whole-cell proteins (10^6 cpm) were mixed with 2–4 μg of eIF-4D or eIF-4C (unlabeled) and analyzed by two-dimensional electrophoresis. (*A*) [^{3}H]Leucine-labeled lymphocyte proteins together with eIF-4D. A radiofluorograph is shown. (*B*) The gel shown in A was stained with Coomassie blue. (*C*) Lymphocyte proteins together with eIF-4C. The Coomassie blue-stained gel is shown. The fluorograph was identical to *A* (data not shown). Hy, Hy^+; 4D, eIF-4D; 4C, eIF-4C; A, actin. M_r markers are $\times 10^{-3}$. In *A*, the dotted circles indicate positions of superimposed eIF-4C and eIF-4D spots in *B* and *C*.

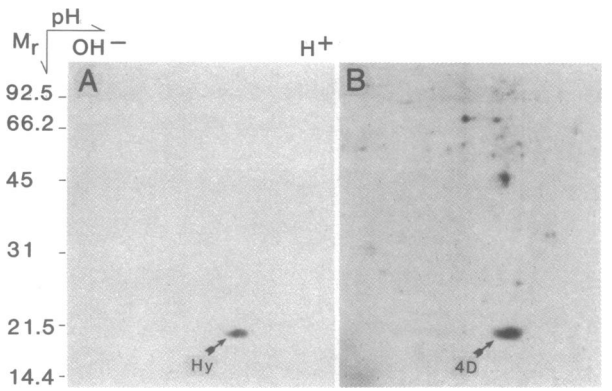

FIG. 3. Comparison of two-dimensional electrophoretic properties of rabbit lymphocyte Hy^+ and eIF-4D. Rabbit peripheral lymphocytes were labeled and analyzed as in Fig. 1 in the presence of eIF-4D. (*A*) Radiofluorograph. (*B*) Coomassie blue-stained gel. Hy, Hy^+; 4D, eIF-4D. M_r markers are $\times 10^{-3}$.

coelectrophoresed with [^{3}H]leucine-labeled lymphocyte proteins. Hy^+ can be identified among newly synthesized lymphocyte proteins by its relative position (4) (Fig. 2*A*) and, in this case also, added eIF-4D migrated to a position that was superimposable on that of Hy^+ (Fig. 2*B*) while eIF-4C gave two stained components, each of higher M_r than Hy^+ (or eIF-4D) (Fig. 2*C*). In Fig. 2*B*, the stained spot representing eIF-4D appears as a double spot. This is a frequent characteristic of Hy^+ (see, e.g., figure 3 of ref. 4) but is probably an artifact of storage.

The two-dimensional electrophoretic mobility of eIF-4D was also compared with that of Hy^+ from rabbit peripheral lymphocytes labeled with [^{3}H]spermidine. Again, only a single peptide was significantly radiolabeled (Fig. 3*A*) and its mobility exactly corresponded with that of eIF-4D (Fig. 3*B*). Our estimate of the pI for Hy^+, based on its position in the isoelectric focusing dimension of two-dimensional electrophoresis, was ≈5.1 (2–4). The reported pI for purified eIF-4D, obtained by more conventional methods, is 6.1 (8). Nevertheless, it is evident from our data that Hy^+ and eIF-4D have the same pI. In our gels, eIF-4D and Hy^+ are slightly more acidic than actin (Fig. 2*A*), which has been reported to have a pI of 5.4 (13). From our gels, we would also assign that pI to actin. Thus, a pI of 5.1 for eIF-4D/Hy^+ is internally consistent.

Our observations imply that eIF-4D and Hy^+ are identical. To verify this conclusion, purified reticulocyte eIF-4D was examined for the presence of hypusine. Acid hydrolysates of eIF-4D analyzed by ion exchange chromatography (12) displayed a peak at the position of hypusine (2–4) (Fig. 4*A*); no peak was detected in a similar chromatogram of eIF-4C (Fig. 4*B*). Other proteins analyzed by this technique also failed to show any component eluting at the position of hypusine (data not shown).

In the chromatographic system used for hypusine detection, arginine is also well resolved from other amino acids (Fig. 4). The relative specific fluorescence of arginine in this system is 60% of the value for hypusine. When allowance is made for this difference, the data in Fig. 4*A* indicate that eIF-4D contains approximately one hypusine residue per five arginine residues. The amino acid composition of purified eIF-4D

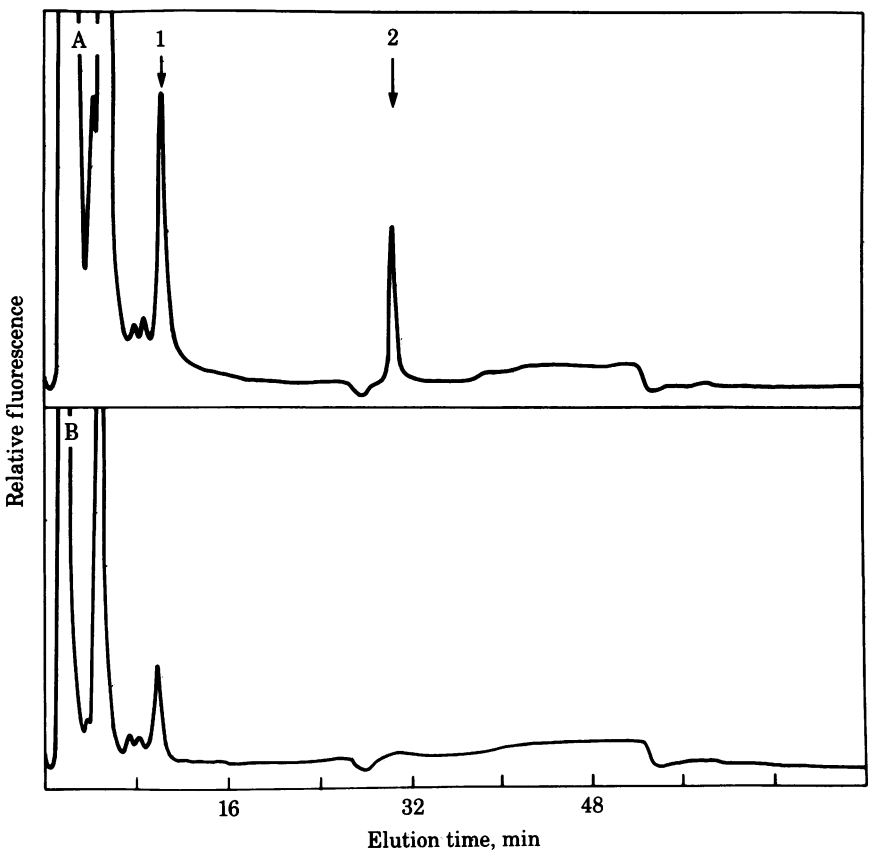

FIG. 4. Chromatographic detection of hypusine in acid hydrolysates of initiation factors. Purified rabbit reticulocyte eIF-4C (*B*) and eIF-4D (*A*) (2.5 μg and 10 μg, respectively) were hydrolyzed with acid (2, 3) and analyzed by an automated ion exchange chromatographic procedure using fluorometric detection (12). Peaks: 1, arginine standard; 2, hypusine standard (2, 3). The base-line depression seen at ≈28 min reflects a change in elution buffer.

(originally designated IF-M2Bα) has been reported (8) and, based on a molecular weight of 17,500, eIF-4D contains approximately five arginine residues per molecule. Thus, eIF-4D contains approximately one hypusine residue per molecule.

DISCUSSION

The protein we have heretofore called "Hy^+" (2–4) has now been shown to have electrophoretic properties, in both isoelectric focusing and $NaDodSO_4$/polyacrylamide gel electrophoresis dimensions, identical to those of the translation initiation factor eIF-4D, purified from reticulocytes (8). Hy^+ was originally studied because it was the only protein detected in growing lymphocytes and other cultured cells that underwent the unusual post-translational modification in which lysine was converted to hypusine (2–4). Analysis of purified eIF-4D revealed that it contains approximately one mol of hypusine per mol of protein. The occurrence of this unusual amino acid, together with the identity of electrophoretic properties, is strong evidence that eIF-4D and Hy^+ are the same protein.

The identical two-dimensional electrophoretic mobility of Hy^+ of human and rabbit origins, reported here, extends our previous similar finding with Hy^+ (henceforth eIF-4D) of human and Chinese hamster origin (4). This is further evidence of the highly conserved structure of this protein.

Of all the translation initiation factors, eIF-4D remains the most problematical, since no definite role has been determined for it in protein synthesis with natural messengers (5, 6). Rather, its activity in cell-free systems is limited to stimulating the formation of ApUpGp-dependent methionylpuromycin and the enhancement of poly(U)-dependent polyphenylalanine synthesis (5, 6). At the present time, no function in intact cells has been identified, and the reality of a physiological role for this factor has been questioned (5, 6). However, since eIF-4D can be detected by Coomassie blue staining, and since we have estimated that only the 10% most prevalent lymphocyte proteins can be so detected (14, 15), it is evident that eIF-4D is, quantitatively, one of the major proteins of the cell. Thomas *et al.* (7) have also found eIF-4D to be, by almost an order of magnitude, the most abundant initiation factor in rabbit reticulocytes. Moreover, the protein is synthesized continuously in resting and growing lymphocytes (4) and a complex site-specific enzyme system is evidently maintained by the growing cell to modify this single protein. These considerations, together with its widespread distribution and structural constancy among animal cells, suggest that eIF-4D plays an important physiological role in intact nucleated cells. The nature of this physiological role is suggested by our earlier data, associating hypusine formation in eIF-4D with accelerating protein synthesis in mitogen-stimulated lymphocytes (4). This leads to the hypothesis that conversion of a lysine residue to hypusine alters the activity of eIF-4D and that the altered activity is involved in regulating the rise in translation rate. Since increased protein synthesis is a major component of activation of quiescent lymphocytes or of growth-arrested fibroblasts, it follows that eIF-4D, and the possible modulation of its activity by post-translational formation of hypusine, may play an essential role in the regulation of cell growth. Post-translational modification as a means of regulating initiation factor activity is well known for eIF-2, for which reversible phosphorylation has been shown to affect function (5). The present work suggests that the post-translational modification of eIF-4D discussed here may be another example of such control.

Our identification of eIF-4D with Hy^+ offers possibilities for the study of this initiation factor in both intact cells and cell-free systems. The factor can be uniquely radiolabeled in its hypusine moiety by growing cells, permitting its identification in various subcellular locations and during binding reactions. As we have shown previously, the peptide can easily be identified by two-dimensional electrophoresis among proteins prepared from subcellular fractions or whole-cell lysates of cells labeled with radioactive amino acids (Fig. 2) (4). This permits studies of relative synthesis and turnover rates in intact cells, as well as possible evaluation of interactions with other proteins. It is to be expected that such studies will lead to improved understanding of the role of this initiation factor in translation generally and in the regulation of cell growth activation in particular.

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