Eukaryotic Initiation Factor 4D

PURIFICATION FROM HUMAN RED BLOOD CELLS AND THE SEQUENCE OF AMINO ACIDS AROUND ITS SINGLE HYPUSINE RESIDUE*

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Eukaryotic initiation factor 4D (eIF-4D) was purified from human red blood cells by a simple 5-step procedure. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that most of the preparations of eIF-4D were composed of variable amounts of two closely migrating forms of the factor, each of which contained a single residue of the unique amino acid hypusine. The structural similarity of the two forms of human eIF-4D was evidenced by the indistinguishable patterns of radioactivity on peptide maps of tryptic digests prepared from radioiodinated samples. A peptide containing the single hypusine residue was readily isolated from a tryptic digest of human eIF-4D by virtue of its high positive charge and hydrophilic character. Amino acid sequence determination on this peptide revealed the following primary structure around hypusine: Thr-Gly-hypusine-His-Gly-His-Ala-Lys.

Experimental Procedures

Materials—Human red blood cells were purchased from the American Red Cross. Chinese hamster ovary cells WTB were kindly supplied by Dr. April Robbins (National Institutes of Health). 201H (carrier-free) and [2,3-3H]putrescine-2HCl (>15 Ci/mmol) were obtained from New England Nuclear. Other materials and reagents are described in earlier publications (6-8, 11, 12).

Methods—Ion exchange chromatographic determination of hypusine was carried out as described earlier (6) with the use of a Dionex D-400 analyzer, the three-buffer system, and fluorometric detection. In this system, the majority of neutral and acidic amino acids are eluted in the breakthrough volume.

Preparation from CHO cells of a radiolabeled eIF-4D fraction for use as a tracer in the preparation of the human red blood cell factor was carried out essentially as described earlier (9), except that cholesterol was not included in the culture medium during growth of the cells. The 40-70% ammonium sulfate fraction prepared from the cell lysate and employed as the tracer was found to contain essentially all of its radioactivity in the form of hypusine in a single 17,000-dalton protein. The specific radioactivity of hypusine in a typical preparation was found to be 5 × 10⁻⁶ cpm/nmol.

Purification of eIF-4D from Human Erythrocytes—Indated or out-dated red blood cells from citrate/phosphate/dextrose human blood were washed twice by suspension in 5 volumes of phosphate-buffered saline and centrifugation at 2000 × g for 5 min. The cells (600 ml of packed cells) were lysed in 2 liters of ice-cold water containing 1 mM dithiothreitol and 0.1 mM ethylenediaminetetraacetic acid. The hemolysate was centrifuged at 25,000 × g for 30 min to remove debris. This and all further operations were conducted at 0–4 °C. All solutions used in further steps of purification contained dithiothreitol and ethylenediaminetetraacetic acid at the concentrations given above. The supernatant was combined with the damp filter cake from 100 g of DEAE-cellulose powder (DE52, Reevaluate) that had been pre-swollen with lysis solution. The mixture was gently stirred for 2 h after which the adsorbent was collected and washed on a Bøchner funnel with 10 liters of 60 mM Tris acetate buffer, pH 6.8. The washed adsorbent was packed into a 5-cm-wide column and further rinsed with 1 liter of the same Tris acetate buffer containing 0.1 M KCl. Elution was conducted using this buffer, but with 0.35 M KCl. Those fractions containing radioactivity were combined.

Ammonium Sulfate Fractionation—Solid ammonium sulfate (0.243 g/ml) was added slowly with stirring. The precipitate collected after equilibration (90 min) and centrifugation (25,000 × g for 20 min) was discarded. To the supernatant was added solid ammonium sulfate (0.205 g/ml). The precipitate obtained was dissolved in 5–10 ml of 0.15 M ammonium acetate buffer, pH 7.8, and the small amount of insoluble material was removed by centrifugation at 25,000 × g.

Exclusion Chromatography on Sephadryl S-200—This solution, which contained about one-half of the original amount of radioactiv-
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RESULTS

The single cellular protein that is radiolabeled when human peripheral lymphocytes or CHO cells are cultured in the presence of [2,3-3H]putrescine or [terminal methylene-3H] spermidine is eIF-4D (5, 6, 11). The labeling of this specific protein results from production of 1 residue of hypusine, the biosynthesis of which involves transfer of the radioactive butylamine moiety of the polyamine spermidine to the ε-amino group of a specific lysine residue (7). Since various mammalian forms of eIF-4D exhibit identical PI values and molecular weights (11), we were able to utilize factor that was purified by the procedure described here. The broken arrow and the solid arrow show the positions of the slower and the faster migrating protein components, respectively. Lane E contains the following molecular weight standards (Pharmacia): phosphorylase b, bovine albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, α-lactalbumin. Electrophoresis was performed on slabs gels by the procedure of Laemmli (15). The acrylamide concentration of the running gels was 12.5%. Prior to application, the samples were made 2% in sodium dodecyl sulfate, 10% in glycerol, and 1% in dithiothreitol, and heated at 100°C for 3 min. Gels were stained with Coomassie Brilliant Blue.

![Fig. 2. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate of eIF-4D. Lanes A, B, and C show the patterns for purified human red blood cell eIF-4D, preparation numbers 16, 17, and 18, respectively; Lane D that for rabbit reticulocyte eIF-4D purified by the procedure described here. The broken arrow and the solid arrow show the positions of the slower and the faster migrating protein components, respectively. Lane E contains the following molecular weight standards (Pharmacia): phosphorylase b, bovine albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, α-lactalbumin. Electrophoresis was performed on slabs gels by the procedure of Laemmli (15). The acrylamide concentration of the running gels was 12.5%. Prior to application, the samples were made 2% in sodium dodecyl sulfate, 10% in glycerol, and 1% in dithiothreitol, and heated at 100°C for 3 min. Gels were stained with Coomassie Brilliant Blue.](image-url)

**Fig. 1.** Chromatography of human red blood cell eIF-4D on (A) Sephacryl S-200 and on (B) phosphocellulose. Conditions for chromatography are given in the text. Fractions of 4 ml were collected and those fractions indicated by the horizontal arrows were pooled.

**Table 1**

| Purification step | Total protein (mg) | Total cpm (x10^5) | Specific radioactivity (cpm/mg) | Yield % |
|-------------------|--------------------|-------------------|--------------------------------|--------|
| Crude lysate      | 1.3 x 10^6         | 10.48             | 8                              |        |
| DEAE-cellulose    | 355                | 5.41              | 1.52 x 10^5                    | 51.6   |
| (NH₄)₂SO₄         | 145.4              | 5.2               | 3.56 x 10^5                    | 49.6   |
| Sephacryl S-200   | 9.9                | 4.3               | 4.2 x 10^4                     | 45.5   |
| Phosphocellulose  | 2.63               | 3.73              | 1.42 x 10^5                    | 35.6   |
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Fig. 3. Patterns of radioactivity on peptide maps prepared from tryptic digests of the radioiodinated slower moving and faster moving components (A and B, respectively) from a sample of purified human red blood cell eIF-4D. The small samples of the two components from preparation number 18 to be used for analysis were isolated by the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in Fig. 2. After staining the gel, the areas containing the components were excised and washed extensively with 10% methanol. Radioiodination, tryptic digestion, preparation of peptide maps, and autoradiography were carried out as outlined in detail earlier (11). The marker compounds, N\(^{-}-(2,4\text{-dinitrophenyl})\text{lysine (DNP-Lys)}\) and N\(^{-}-(5\text{-dimethylaminonaphthalene-1-sulfonyl})\text{cadaverine (DC)}\), were applied along with the samples. The peptide denoted by the broken arrow is discussed in the text.

Fig. 4. Reverse phase high performance liquid chromatographic pattern of tryptic peptides from human red blood cell eIF-4D. Tryptic digestion of eIF-4D, preparation number 16 (0.5 mg/ml), was conducted with trypsin at a level of 20 \(\mu\text{g/ml}\) using other conditions as outlined earlier (11). The water and buffer salt were removed by lyophilization and the residue was dissolved at \(\frac{1}{3}\) the original volume in the starting mobile phase used for chromatography. Chromatography was performed on the digest from 0.2 mg of protein with the use of a 0.39\(\times\)30-cm column of \(\mu\)Bondapak C\(_18\) (Waters Associates) and a gradient as shown between 0.1% (v/v) trifluoroacetic acid and acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The flow rate was 1 ml/min and monitoring was at 210 nm with a full scale absorbance of 1.0. The hypusine-containing peptide, denoted by the vertical arrow, was obtained in \(\sim 70\%\) yield as estimated from recovery of radioactivity.

The Coomassie Blue-stained bands from several different preparations of red blood cell eIF-4D were excited from gels and washed well with 10% methanol. Treatment of each of the gel pieces with trypsin and separation of digestion products from the insoluble gel material was carried out as outlined earlier (11). Following hydrolysis of the digestion products with 6 N HCl, the amount of hypusine was estimated fluorometrically after its separation by ion exchange chromatography. It was necessary to separate hypusine-containing peptide material from the gel pieces before acid hydrolysis in order to avoid formation from the gel constituents of compounds that caused false response in the fluorometric determination of hypusine. In those samples of red blood cell eIF-4D that displayed the two-band staining pattern, hypusine was found to be a component of the protein in each of the bands. In each sample, the content of hypusine in the protein from the two gel positions was approximately the same as judged from the band staining intensities and from the fluorescent intensities of the breakthrough peaks of amino acids in the chromatograms. Surprisingly, the radiolabeled CHO cell eIF-4D which was initially added as a tracer was found to co-migrate with only one of the protein components of those eIF-4D preparations that exhibited the two band pattern. Radioactivity
TABLE II

**Analysis of the hypusine-containing peptide from human red blood cell eIF-4D**

| Amino acid | Residues/mol |
|------------|--------------|
| Thr        | 1.3          |
| Gly        | 2.2          |
| Ala        | 1.1          |
| His        | 1.9          |
| Lys        | 1.0          |
| Hypusine   | 1.0          |

*Calculated based on 1 residue of hypusine/mol and normalized to the value of 1 for hypusine. For sequence analysis, yield based on Gly 2 = 76%, repeated yield = 98%.  
*Identified as hypusine from the release of radioactivity at this step of degradation (11).

*NI, not identified.

| Cycle | Automated method | Manual method |
|-------|------------------|---------------|
| n mol |                  |               |
| 1     | Thr 0.19         | Thr           |
| 2     | Gly 0.51         | Gly           |
| 3     | Hypusineb        | Hypusineb     |
| 4     | His 0.24         | His           |
| 5     | Gly 0.49         | Gly           |
| 6     | His 0.23         | NF            |
| 7     | Ala 0.24         | NI            |
| 8     | Lys 0.18         | NI            |

were detected only in the faster moving component (solid arrow) both upon examination by autoradiography or by direct measurement in digests of the isolated bands.

Since both proteins in the samples of purified eIF-4D were found to contain hypusine, it was of interest to examine further their structural relationships. The striking similarity in the patterns of radioactive peptides on maps prepared from tryptic digests of radiolabeled samples of the proteins from the two gel positions is seen in Fig. 3. The peptide denoted in each map by the broken arrow warrants special attention. This peptide occupies the approximate position of the hypusine-containing tryptic peptide from eIF-4D of several mammalian cells (11). The likelihood that it is indeed this peptide from each of the two protein components of human red blood cell eIF-4D becomes more evident with the knowledge that this peptide contains amino acid residues, *i.e.* 2 histidine residues (see below), that become radiolabeled under the experimental conditions used for radioiodination.

From the unique position occupied by the hypusine-containing peptide on the tryptic maps of Fig. 3, it was speculated that this peptide is strongly positively charged and very hydrophilic and should be quite easily isolated in quantity from the bulk of tryptic peptides. This indeed proved to be the case. Fig. 4 shows the reverse phase high performance liquid chromatographic pattern of the tryptic peptides of red blood cell eIF-4D. The hypusine-containing peptide, designated by the vertical arrow, was eluted rapidly from the column, well separated from all other peptides. In Table II are given the amino acid composition of, and the sequence data on, this peptide. These results are in close agreement and allow us to postulate the sequence as: Thr-Gly-hypusine-His-Gly-His-Ala-Lys.

**DISCUSSION**

The procedure for isolation of eIF-4D polypeptide outlined here provides a large-fold purification in good yield from a plentiful human source. The procedure is new in that specifically biosynthetically radiolabeled factor, prepared in cultured cells from another mammalian source, was employed to follow steps in purification. The decision to purify using this means of assay was based on our earlier evidence for very similar physical properties and a large degree of homology in this protein from various mammalian sources (11). In light of the complexity of the biological assay for eIF-4D, and because of the lack of knowledge as to its specific physiological function, the simple radiotrace procedure seemed appropriate for isolation of this hypusine-containing factor. Indeed, this purified material should be most useful for physical and chemical studies.

Antibodies prepared to the isolated factor should provide a means for comprehensive study of the physiological role of eIF-4D. Together with knowledge of the primary structure of this factor, the first information on which is included in this report, should come some understanding of the function of the unique hypusine residue, and, ultimately, the importance of this post-translational modification to which the polyamine spermidine contributes a structural part. It is tempting to speculate that the highly positively charged hydrophilic region of sequence around hypusine bears a special role in interactions between eIF-4D and ribosomes, nucleic acids, and/or acidic domains in other proteins. Certainly, knowledge of this sequence confirms our earlier suggestions that eIF-4D contains 1 single residue of hypusine (5, 11). It supplies a glimmer of the basis for specificity of the biosynthesis of hypusine and provides a preliminary pattern for construction of oligonucleotide probes, and of peptide substrates and inhibitors for the enzymes that promote this unique post-translational event.

Biosynthetic labeling of hypusine in eIF-4D has provided strong evidence for a single form of eIF-4D in cultured cells (5, 6). Therefore, the finding of varying amounts of two forms of eIF-4D in human red blood cell preparations was surprising. However, multiple molecular weight forms of certain other initiation factors have been isolated from rabbit reticulocytes (10). The relative amounts of these forms were reported to vary considerably from one preparation to another, probably as a result of limited proteolysis in vivo and/or during isolation of the factors. In our preparations, the radiolabeled tracer from CHO cells does not change during the isolation procedure and radioactivity is consistently found associated with one form only of the human red cell eIF-4D. There are several possible causes for this finding. (i) The native form of human eIF-4D is identical with the tracer CHO cell eIF-4D. In *vivo* or during storage of the red cells there is partial conversion to the second form, but, after addition of tracer to cell lysate, and during the subsequent purification, there is no further change. (ii) The native form of red cell eIF-4D displays the identical migration in sodium dodecyl sulfate-polyacrylamide gel electrophoresis as tracer factor. However, because they originate from separate mammalian species, the two factors contain one or more differences in their primary structures. During storage and/or isolation, the red blood cell eIF-4D is partially converted to a new slower migrating form, whereas the CHO cell radioactive tracer eIF-4D is not. This differential change occurs because the chemical modification that
causes this conversion is limited to a region of primary structure that exists exclusively in the red blood cell factor. (iii) The native form of human eIF-4D is that which migrates more slowly than the radioactive tracer. In vivo, during storage, and/or during purification, there is a partial or complete conversion to a form that migrates with the tracer eIF-4D. (iv) Finally, there may exist two genetically distinct forms of human eIF-4D.

At present we have no strong reason to favor one of these causes for association of tracer radioactivity with one form only of red blood cell eIF-4D. We have seen no consistent relationship between the ratios of the two forms in eIF-4D preparations and the times or conditions of storage of whole human blood or red cells. It is interesting that after treatment of a sample of the red blood cell factor that displays one band of a substantially higher level of methionine in preparation number 16, which displays a single component that migrates with tracer, than in preparation number 18 in which there is a majority of the slower moving form. Whether a conversion of one form of eIF-4D to another that may occur in red blood cells in vivo or during aging or storage is of biological significance in terms of factor inactivation or degradation is not known. This, together with the structural differences in the two forms of human red blood cell eIF-4D, is under investigation.

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