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Evidence That the Human Homologue of a Rat Initiation Factor-2 Associated Protein (p^{67}) Is a Methionine Aminopeptidase

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Previously, we cloned a human cDNA encoding a protein which has a 92% amino acid sequence identity to a rat initiation factor-2 associated protein (p^{67}). Rat p^{67} plays an important role in translational regulation by preventing the phosphorylation of the α subunit of initiation factor-2. Interestingly, several lines of indirect evidence suggested that this protein may also function as a methionine aminopeptidase (MetAP). To test this hypothesis, we expressed the human cDNA in a baculovirus system, purified it to homogeneity and characterized it. Using 13 different peptide substrates, we found that the human p^{67} has a similar substrate specificity with other MetAPs. Kinetic analyses revealed that the K_{cat}/K_m values of the human MetAP on two representative substrates are similar to those of yeast and porcine MetAPs. Furthermore, we found that this enzyme, like other MetAPs, is also a cobalt-dependent metalloenzyme.

Eukaryotic initiation factor-2 (eIF-2) comprises three subunits: α, β and γ. Phosphorylation of the eIF-2α inactivates eIF-2 activity and inhibits protein synthesis (reviewed in Refs. 1-2). This regulatory mechanism is widely used in animal cells under nutritional deprivation, heat shock, and viral infection. Recently, an eIF-2 associated protein (p^{67}) was isolated and characterized from rabbit reticulocyte lysates (3). This protein can promote protein synthesis in the presence of active eIF-2 kinases by protecting the eIF-2α from eIF-2 kinase-catalyzed phosphorylation (3).

The gene encoding rat p^{67} was cloned (4). Sequence analysis revealed that it contains 480 amino acid residues with a calculated molecular mass of 53 kDa. We recently cloned the gene of its human homologue (5). The human p^{67} is 92% identical to the rat protein in amino acid sequence. In the N-terminal region of both rat and human p^{67}, there are two basic polylysine blocks and one aspartic acid block. These polylysine blocks, which also exist in human eIF-2β and yeast protein Sui3, have been postulated to be involved in protein/protein or protein/nucleic acid interactions (4, 5). Interestingly, the C-terminal region of both human and rat p^{67} shares ~22% identity with both yeast MetAP-1 and *E. coli* MetAP. In addition, the five residues involved in metal binding in *E. coli* MetAP are strictly conserved in both rat and human p^{67} (5-7). These findings led to the proposal that p^{67} may be a methionine aminopeptidase (5).

Herein, we report the expression, purification and characterization of human p^{67}. Our results demonstrated that human p67 has a similar substrate specificity with other MetAPs. This enzyme, like other MetAPs, is also a metalloenzyme, whose activity can be stimulated by Co^{2+} and inhibited by metal chelating regents.

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Abbreviation: MetAP, methionine aminopeptidase.

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MATERIALS AND METHODS

DNA constructions. Two oligonucleotide primers, 5′-GCGAAGCTT(Hind III)ATGTACCCATACGATGTTCCA-GATTACGCTAGCGCGGGTGTGGAGGAGG (forward) and 5′-GGCTCTAGA(Xba I)TTAATAGTCATATCCTCT (reverse) were used to amplify the cDNA of human p^{67}. We introduced the coding sequence of the hemagglutinin epitope (YPYDVPDYA) to the 5′-end of the gene so that the 12CA5 monoclonal antibodies against the hemagglutinin epitope can be used to monitor the expression and purification process of the recombinant human protein. A Hind III site at the 5′-end and an Xba I site at the 3′-end were also generated to facilitate subcloning of the PCR product. After being digested with Hind III/Xba I, the PCR product was inserted into the corresponding sites of plasmid Bluescript. Recombinant plasmids were sequenced by Sanger's dideoxy nucleotide chain termination method. The Hind III/Xba I fragment that has no mutation, was then subcloned into the corresponding sites of the transfer vector pBacPAK8 (Clontech Laboratories, California), resulting in a vector pPAK8-hp67.

Cell culture and generation of recombinant baculovirus. Sf21 insect cells (Clontech) were routinely propagated at 27 °C in TNM-FH medium (Sigma Chemical, Missouri) supplemented with 10 % fetal calf serum and antibiotics (penicillin 50 units/ml and streptomycin 50 μg/ml). Generation of recombinant baculovirus was performed according to the manufacturer's protocol (Clontech). The expression of the recombinant protein was detected by immunoblot analysis as described below.

Immunoblot analysis. Protein samples, obtained from sf21 cells of different infection stages or from different purification steps as described below, were separated by 10% SDS-PAGE (8), and transferred to nitrocellulose filters according to the manufacturer's protocol (Bio-Rad Laboratories, California). The filters were first incubated with the 12CA5 anti-hemagglutinin epitope monoclonal antibodies (Berkeley Co., California) for two hours at room temperature after being blocked overnight with 5 % (w/v) dry milk in TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween- 20) at 4 °C. They were then incubated for one hour with goat anti-mouse IgG conjugated with alkaline phosphatase and developed with the alkaline phosphatase substrates according to manufacturers' instructions (Bio-Rad).

Purification of the recombinant human p^{67} from insect cells. The purification procedure was carried out at 4 °C. According to the immunoblot analysis, expression of human p^{67} reached the highest level at day 4 post-infection. At day 4, cells were collected by mechanical disaggregation from 12 dishes (10 × 1.5 cm) of confluent culture and pelleted at 1500 × g for 2 min. The pellet was washed with physiological saline (0.9 % NaCl) once and resuspended in 6 ml of buffer X [10 mM Hepes, pH 7.4; 0.5 mM $CoCl_2$; 10% Glycerol (v/v)] containing 1.5 mM $MgCl_2$, 100 mM KCl and protease inhibitors (aprotinin, 1 μg/ml; leupeptin, 1 μg/ml; pepstatin A, 0.7 μg/ml; phenylmethylsulfonyl fluoride, 1 mM). Cells were then disrupted manually with a Dounce homogenizer. The homogenate was centrifuged at 10,000 × g for 20 min, and the supernatant was loaded on a 2 ml S-Sepharose column after 1:3 dilution with buffer X containing no salt. The column was first washed with 5 column volumes of buffer X containing 50 mM KCl and the bound proteins were eluted with buffer X containing 0.5 M KCl. Each collected fraction was examined for the recombinant human protein by immunoblot analysis. Fractions containing the human protein were pooled and dialyzed against buffer X containing 50 mM KCl. The sample was then loaded onto a Mono Q column. After washing the column extensively with buffer X containing 50 mM KCl, bound proteins were eluted with a 30-ml linear gradient from 50 mM to 1 M KCl (in buffer X) at 1 ml/min (data not shown). Each fraction (1 ml) was examined for the recombinant human protein as described above. Fraction 11 which contained most recombinant protein was used for further purification. This fraction, after being diluted ten times with buffer X containing no salt, was loaded onto a Mono S column. After washing the column with buffer X containing 50 mM KCl, the bound proteins were eluted with a 30-ml liner gradient from 50 mM to 1 M KCl (in buffer X) at 1 ml/min (data not shown). Each fraction was examined by immunoblot analysis as described above. Fraction 29 contained the homogenous recombinant p^{67}, as shown in Figure 1. This fraction was used for enzyme assays and it remained stable for several months when stored at 4 °C.

M_r determination by gel-filtration chromatography. Gel-filtration chromatography was carried out on a Progel- TSK G3000SW (30 cm × 7.5 mm ID) column (Supelco Co., Pennsylvania). Ten μg of purified human protein was chromatographed in 0.1 M NaH_2PO_4, 0.1 M Na_2SO_4, pH 6.8, 0.05% NaN_3 (w/v) at a flow rate of 0.75 ml/min. The marker proteins used were thyroglobulin (670 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa) and myoglobin (17 kDa). Fractions were collected at 0.5 min intervals and the human protein was located by immunoblot analysis.

In vitro MetAP enzyme assay and effect of divalent cations, EDTA and hydrogen peroxide on MetAP activity. We carried out MetAP enzyme assay as described by Zuo et al. (9). Briefly, various amounts of peptide substrates were added to the enzyme solution and incubated at 37 °C. Aliquots were taken at different time intervals (0, 5, 10 and 15 min) and the reactions were terminated by the addition of EDTA to 10 mM, followed by boiling for five minutes. The concentrations of the released methionine were determined by the AccQ-Tag method (10). To determine the specific activity of MetAP after each purification step, we measured the protein concentrations by Bradford's method (11). For kinetic analyses, we measured the protein concentrations according to the optical density at 280 nm. In the latter case, the molar extinction coefficient (ϵ_{280nm} = 46,700) of the recombinant human protein was calculated using

TABLE 1
Purification of the Recombinant Human MetAP

Step	Total protein mg	Total activity[a] units[b]	Specific activity[a] units/mg	Purification -fold	Yield %
Crude extract	5.26	6.27	1.19	—	—
S-Sepharose	1.09	0.493	0.453	1	100
Mono Q	0.06	0.297	4.95	10.9	60.3
Mono S	0.015	0.171	11.57	25.6	34.8

[a] MetAP activity was determined using substrate Met-Ala-Ser (2 mM).
[b] 1 unit was defined as the amount of protein which releases 1 μmol of Met per min at 37°C.

the Peptidesort program from the GCG package version 8 (Genetics Computer Group, Wisconsin). All kinetic data were analyzed using SigmaPlot program (Jandel Scientific, California). Kinetic parameters were calculated from double-reciprocal (Lineweaver-Burk) plots.

To determine the effect of divalent cations, EDTA or hydrogen peroxide on MetAP activity, the human enzyme samples which contained 0.5 mM Co^{2+} were diluted 400-fold in buffer X containing no Co^{2+}, resulting in a [Co^{2+}] of 1.25 μM. EDTA, H_2O_2 or different divalent ions were added, respectively, to the MetAP solution and incubated at 4 °C for 15 min. After the assay solutions were warmed to 37 °C, reactions were started by adding peptide Met-Ala-Ser to 2 mM. The released methionine was quantified at different time points (0, 10, 20 and 40 min) (10). In the second study, the metal ions in the protein solution were removed by extensive dialysis against a buffer containing 1 mM EDTA; 10 mM Hepes, pH 7.4; 100 mM KCl; 10% (v/v) glycerol at 4 °C. EDTA was removed by further dialysis against the same buffer without EDTA. Different cations were added and assayed as described above.

RESULTS

Baculovirus Expression of the Recombinant Human p67 and Its Purification

We used the baculovirus system to express the human p67 cDNA and developed a three-step purification procedure, which includes two anion-exchange columns and one cation-exchange column. As summarized in Table 1, this procedure produced a 25.6-fold purification with a 34.8% yield, assuming the -fold of purification is 1 and the recovery is 100% for the first purification step. We used this assumption because it is difficult to determine the amount of this enzyme in crude extract due to the presence of many other non-specific aminopeptidases in insect cells. After the final purification step, the protein was illustrated as a single band by SDS-PAGE (Figure 1). The molecular mass of the human protein was estimated to be ~65

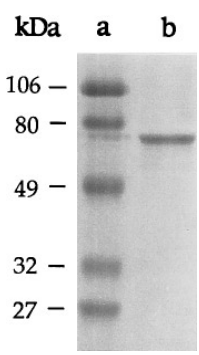

FIG. 1. SDS-polyacrylamide gel electrophoresis of the purified recombinant human p^{67}. About 2 μg of purified human p^{67} was loaded on a 10% SDS-polyacrylamide gel and electrophoresis was performed, followed by Coomassie blue staining. Lane a, pre-stained molecular weight standards: phosphorylase B (106 kDa), bovine serum albumin (80 kDa), ovalbumin (49 kDa), carbonic anhydrase (32 kDa), soybean trypsin inhibitor (27 kDa). Lane b, recombinant human p^{67}.

TABLE 2
Relative Activities of the Recombinant Human MetAP among Various Substrates

Substrate	Activity (%)
Xaa-Ala-Ser[a]	0
Met-Ala-Ser	100 ± 19
Met-Gly-Met-Met	119 ± 15
Met-Pro-Gly	39 ± 3.9
Met-Ser-Gly	85 ± 10.6
Met-Leu-Gly	0
Met-Met-Ala	0
Met-Arg-Phe-Ala	0
Met-Tyr-Lys	0

The enzyme activity was determined using 2 mM substrate under standard assay conditions. Activities are presented relative to substrate Met-Ala-Ser. Data are reported as mean activity ± S.D. (n = 2–4).

[a] Xaa, Ala, Glu, Ile, Gln, Ser.

kDa by gel filtration (data not shown) and ~70 kDa by SDS-PAGE. This indicates the human p67 is a monomer.

During the development of the purification procedure, we found that the recombinant protein binds to both anion exchange (Mono-Q) and cation exchange (Mono-S) columns at pH 7.4. It is predictable that this human protein should bind to a Mono-Q column at pH 7.4 since its calculated pI is 5.64. However, it is unusual that it also binds to a Mono-S column at pH 7.4. There are at least two possible factors that may contribute to this property. First, since cobalt ions bind Mono-S resins tightly, a Mono-S column pre-equilibrated with a buffer containing Co^{2+} may act as an affinity column for cobalt binding proteins, such as MetAPs. Second, human p^{67} contains two highly positively charged regions which may also contribute partially to the binding of the Mono-S column. The purified recombinant human protein, like rat p^{67}, has a larger estimated M_r (~70 kDa) than its calculated M_r (~54 kDa). This discrepancy may be caused by post-translation modification(s) or abnormal binding ratio to SDS. The size of the human p^{67} is very similar to that of porcine MetAP (15), even though their amino acid compositions were very different (data not shown).

Substrate Specificity

To determine whether human p^{67} is a MetAP, we used two groups of substrates for enzyme assays (Table 2). Among the first group of substrates, Xaa-Ala-Ser (Xaa: Met, Ala, Gln, Glu, Ile or Ser), human p^{67} showed activity only on Met-Ala-Ser. These results indicate that human p^{67} is an aminopeptidase that prefers peptide substrates with an NH_2-Met. This distinguished itself from other non-specific aminopeptidases, such as Leu-aminopeptidase, aminopeptidase N, and aminopeptidase-P (12-14). Among the second group of peptide substrates, the human MetAP cleaved N-terminal methionine from peptide substrates only when the penultimate residue is small and uncharged (Ala, Gly, Pro, Ser). This is generally in agreement with the specificity of the MetAPs from bacteria, yeast and porcine liver. Thus, we conclude that human p^{67} is a MetAP. The k_{cat} and k_m values of the purified enzyme on two representative substrates were determined and shown in Table 3. These values are very similar with those of the porcine MetAP and yeast MetAP1 (15, 16).

TABLE 3
Kinetic Parameters of the Recombinant Human MetAP

Substrate	Km (mM)	Kcat (min^{-1})	Kcat/Km (mM^{-1} min^{-1})
Met-Ala-Ser	0.670 ± 0.045	930 ± 67	1388 ± 100
Met-Gly-Met-Met	0.646 ± 0.021	1087 ± 89	1683 ± 138

The kinetic parameters of the recombinant human MetAP were determined under standard conditions using substrate concentrations from 0.1 to 2 mM. The values were deduced from reciprocal plot. Data are reported as mean values ± S.D. (n = 3).

Metal Ion Requirement for MetAP Activity

Since all MetAPs have been shown to be Co^{2+}-dependent metalloproteases, we tested whether the human MetAP is also a metalloenzyme. As shown in Table 4, the human MetAP activity increased to 2-fold when the cobalt concentration was increased from 1.25 μM to 0.1 mM. Zinc ion, EDTA and H_2O_2 showed inhibitory effects. In the presence of 3 mM zinc, human MetAP is completely inactivated, but porcine MetAP is still ~30% active (15), indicating that human MetAP is more sensitive to zinc ions than porcine MetAP. In the presence of 1 mM EDTA, the human MetAP showed significant activity. However, extensive dialysis against EDTA-containing buffer totally inactivated human MetAP, and its activity can be recovered to 77 % by 0.1 mM Co^{2+} or to 47% by 3 mM of Mn^{2+} (Table 5). The effect of Mn^{2+} was surprising as such effect has not been observed for any other characterized MetAPs.

Miller and Schimmel reported that enzyme-cobalt coordination can be altered by oxidation of Co^{2+} to Co^{3+} *in situ* with hydrogen peroxide (18). Accordingly, we examined the Effect of H_2O_2 on MetAP activity. Under our assay conditions, human MetAP activity was decreased to 50% in the presence of 50 μM of H_2O_2. It is possible that part of the bound Co^{2+} was

TABLE 4
Effect of Divalent Cations, EDTA and H_2O_2 on the Human MetAP Activities

Treatment		Activity (%)
$CoCl_2$	0.1 mM	185
	0.5 mM	182
$MgCl_2$	0.5 mM	91
	3.0 mM	94
$MnCl_2$	0.5 mM	107
	3.0 mM	93
$ZnCl_2$	0.5 mM	4.7
	3.0 mM	0
EDTA	1.0 mM	66
	5.0 mM	45
H_2O_2	10.0 μM	75
	50.0 μM	50

Activities were determined using substrate Met-Ala-Ser (2 mM) under conditions described in the text. Control samples assayed under same conditions (with no additions) were considered as 100%. Each value represents the average of duplicates.

TABLE 5
Partial Recovery of MetAP Activity by Cobalt and Manganese after EDTA-Treatment

Treatment		Activity (%)
Before dialysis		100
After dialysis		0.3
+ $CoCl_2$	0.1 mM	77.4
	0.5 mM	75.6
+ $MgCl_2$	0.5 mM	0.73
	3.0 mM	0.83
+ $MnCl_2$	0.5 mM	37.9
	3.0 mM	47.0
+ $ZnCl_2$	0.5 mM	0.45
	3.0 mM	0.13

Activities were determined using substrate Met-Ala-Ser (2 mM) under standard conditions. Each value represents the average of duplicates. The original enzyme sample before dialysis is considered as 100%.

oxidized by hydrogen peroxide to Co^{3+} in the human MetAP, which in turn partially inactivated the enzyme.

DISCUSSION

Removal of the initiation methionine is important for the normal function of many proteins (19-21). Examples include hemoglobin and some proteins involved in signal transduction, protein trafficking, cancer cell growth, and viral infection (22, 23). Prokaryotic methionine aminopeptidase (MetAP) genes from *E. coli* and *S. typhimurium* have been cloned and sequenced (24, 25). Deletion of these bacterial MetAP genes was lethal, indicating that MetAP is essential for bacterial growth (26, 27). It has also been shown that yeast require two distinct MetAPs to provide the essential function that can only be partially provided by MetAP1 or MetAP2 alone (28). Yet, very little is known about the properties of human MetAP (29). Here we provide for the first time the direct evidence that the human p^{67} is a MetAP. This finding strongly suggests that this protein has dual functions: a p^{67}-like function in regulation of protein synthesis and a MetAP-like function in amino-terminal protein processing.

Kinetic analyses revealed that the human MetAP has similar kinetic parameters with those of porcine MetAP as well as yeast MetAP1 (15, 17). The K_m values of human MetAP on its peptide substrates are $\sim$0.6 mM, wheras the K_m value of other enzymes involved in amino-terminal protein processing, such as N-acetyltransferases and N-myristoyltransferases, are in micromolar ranges (23, 30). The low affinity of MetAPs to the peptide substrates led us to propose that ribosomal association may be important for MetAP to cleave the initiator methionine effectively *in vivo* (16). The rat p^{67} has been shown to be associated with eIF-2 which carries the Met-tRNA to ribosomes. If human MetAP associates with eIF-2, like rat p^{67}, it may use this mechanism to bind to ribosomes so that it can effectively process newly synthesized proteins.

Many aminopeptidases have been found to be metalloproteases (31, 32). Examples include Leu-aminopeptidase from bovine eye lens which contains two zinc ions in the active site, aminopeptidase-P which requires Mn^{2+} for its activity, and all known MetAPs that require Co^{2+} for catalysis (15, 17, 24, 25). The human MetAP, like other MetAPs, is also a metallopro-

tease. In the presence of 1 mM EDTA human MetAP still showed significant activity (Table 4). It was totally inactivated only after extensive dialysis against EDTA (Table 5). Since human MetAP contains all the five conservative cobalt-binding residues that were identified in *E. coli* MetAP, it is reasonable to assume that human MetAP, like *E. coli* MetAP, also contains two cobalt ions. If so, one possible explanation for the EDTA effect is that human MetAP may have one loosely bound cobalt and one tightly bound cobalt. The loosely bound cobalt can be readily removed by EDTA which results in decreased enzyme activity; the tightly bound one, on the other hand, can only be removed after extensive dialysis against EDTA which results in total inactivation of the enzyme.

It is worth noting that Dr. Gupta and his coworkers found that rat p67 is highly regulated (3). During heme deprivation in reticulocyte or in serum-starved animal cells in culture, p^{67} is degraded, which results in the phosphorylation of eIF-2α and the inhibition of protein synthesis. They also found that addition of a mitogen, phorbol 12-myristate 13-acetate, to serum-starved cells in culture can induce the expression of p^{67} with accompanying increase in protein synthesis. Our finding that p^{67} is a MetAP raised the question whether N-terminal processing is also regulated in a similar manner and how it is related to cell growth. We are currently actively engaged in this study.

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