Mutations in the NKXD consensus element indicate that GTP binds to the γ-subunit of translation initiation factor eIF2

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Abstract Initiation factor eIF2 binds GTP and promotes the binding of methionyl-tRNA to ribosomes. Biochemical and sequence evidence suggests that the GTP might bind to either the β- or γ-subunit of eIF2. Mutations were made in the NKXD consensus elements found in both subunits and individual mutant forms were overexpressed in transiently transfected COS-1 cells. The effect on the translational efficiency of a reporter mRNA for dihydrofolate reductase was monitored. Mutations in the γ-subunit cause severe repression of protein synthesis, whereas those in the β-subunit are only mildly inhibitory. The results support the view that GTP binds exclusively to the γ-subunit.

Key words: Protein synthesis; eIF2; GTP binding; Mutagenesis

1. Introduction

Translation initiation factor eIF2 catalyzes the GTP-dependent binding of Met-tRNA$_i$ to the 40S ribosomal subunit. After hydrolysis of GTP bound to eIF2, an eIF2-GDP binary complex is released from the ribosome and subsequently GTP is exchanged for GDP in a reaction catalyzed by eIF2B. eIF2 is a complex comprised of three nonidentical subunits: α (36 kDa); β (37 kDa) and γ (52 kDa). The roles of the individual subunits in the function of eIF2 are not entirely clear and evidence identifying the subunit responsible for binding the guanine nucleotide is contradictory. In an early report, denatured eIF2 subunits were separated and the α-subunit was found to bind GTP [1]. In contrast, affinity labeling of eIF2 with GTP derivatives occurs both to the β- and γ-subunits [2,3]. When the cDNAs encoding human eIF2 were cloned and sequenced, the DXXG and NKXD consensus elements for GTP binding were found in both the β- and γ-subunits [4,5], reinforcing the possibility that both subunits might be involved. To address this problem directly, we have mutated the asparagine residue in the NKXD consensus element of both subunits and have tested the in vivo activities of the mutant forms by overexpression in transiently transfected COS-1 cells.

2. Materials and methods

2.1. Site-directed mutagenesis

Point mutations in the Asn codon in the NKXD consensus elements of the β- and γ-subunits were prepared by using the 'Altered Sites' in vitro mutagenesis system (Promega Corp.). For the β-subunit, the 1.1 kb *Eco*RI/*Hin*dIII fragment from pGEM2–2β [4] was subcloned into the pAlter-1 mutagenesis vector and mutated with the mixed oligonucleotide 5′-GATGAAGAC(T,C,G)ACAAAAAAGATG-3′. Plasmids carrying the mutation were identified by dideoxy sequencing with the primer 5′-CTAACCAGAGGATGACC-3′ (Sequenase Kit, US Biochemicals Corp.). Similarly, the corresponding residue, Asn^{190}, in the γ-subunit was altered by subcloning a 1.7 kb *Eco*RI fragment from pSP72–2γ [5] into pAlter-1, mutating with 5′-GATTCTACAA-(T,C,G)ATAAAATTGATTTGG-3′ and sequencing with 5′-GGCTA-CTATGCTGAACGGTGC-3′.

2.2. Expression of eIF2β and eIF2γ

Mutated DNAs encoding eIF2β were excised from the pAlter-1 vector by digestion with *Eco*RI and *Hin*dIII, blunt-ended with Klenow fragment and cloned into the blunt-ended *Eco*RI site of pMT3 [6]. Mutated eIF2γ DNAs were excised from the pAlter-1 vector by digestion with *Eco*RI and subcloned into the *Eco*RI site of pMT3. The proper orientation of all inserts was confirmed by restriction analysis. The resulting pMT3–2β and pMT3–2γ plasmids were individually cotransfected with the pMT3 vector into COS-1 cells by the DEAE-dextran method [6]. Cells were labeled with [^{35}S]Met (1000 Ci/mmol, Amersham Corp.) at 40–48 h posttransfection, washed and scraped from plates. Half of the cells were pelleted and lysed directly into SDS gel sample buffer [7], while the other half were lysed in 10 mM HEPES pH7.7, 150 mM NaCl, 5 mM $MgCl_2$, 10 mM vanadyl ribonucleoside complex (New England Biolabs) and 0.3% Nonidet-P-40 for RNA analyses.

3. Results

To distinguish the contribution to GTP binding of the NKXD consensus element in the β- and γ-subunits of eIF2, the Asn residue was altered by site-directed mutagenesis as described in section 2 and depicted in Fig. 1A. X-ray crystallographic models of the G-domain of EF-Tu and *ras* p21 suggest that the N and D residues hydrogen bond to the guanine ring (Fig. 1B) [8–10]. Substitution of this Asn residue with tyrosine causes loss of GTP/GDP binding activity and profoundly affects the biological property of *ras* p21 [11]. We therefore focused our attention on this residue, assuming that the corresponding change in eIF2 would also prove detrimental to GTP binding and eIF2 function.

The mutagenesis protocol generated replacements of Asn by Tyr (Y) and Asp (D) for eIF2β and Tyr (Y), Asp (D) and His (H) for eIF2γ. Each of the mutant forms of the two subunits was evaluated for in vivo function by cotransfections with pMT3 which expresses the reporter protein, dihydrofolate reductase (DHFR), as described in section 2. The pMT3–2β and pMT3–2γ plasmids express the subunits from the adenovirus major late promoter and SV40 enhancer.

Overproduction of eIF2 subunits was monitored by SDS-PAGE and immunoblot analysis at 40 to 48 h posttransfection. The wildtype and mutant eIF2β bands are 5- to 10-fold more intense than those in mock-transfected cells (Fig. 2). Similar results (not shown) were obtained for eIF2γ, as reported previously [5]. The immunoreactive bands were quantitated by scanning and are reported in Table 1. Since only 10–20% of the cells

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SSDI 0014-5793(95)00993-0

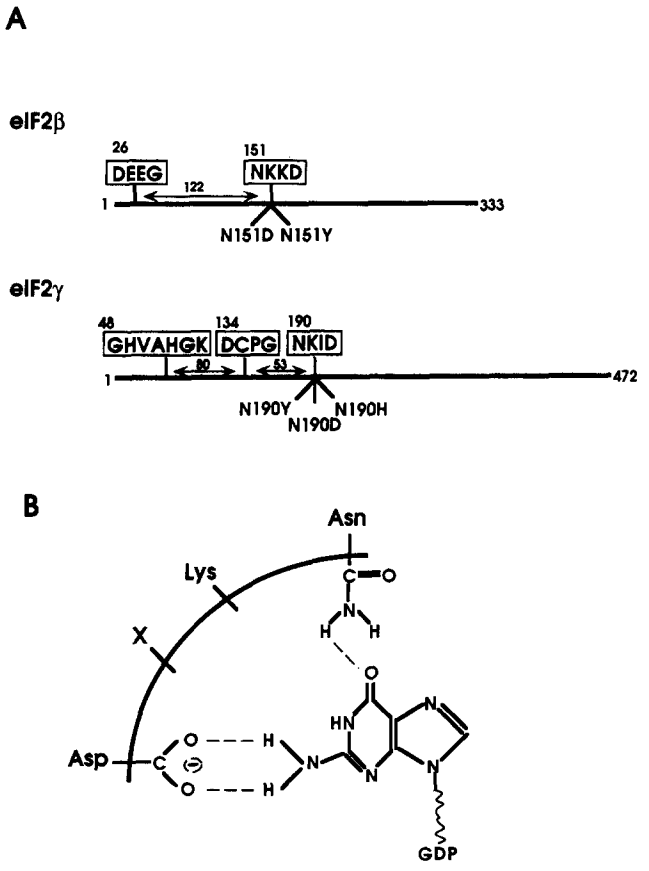

Fig. 1. Schematic structures of eIF2β and eIF2γ. (A) The structures of the two subunits are shown by horizontal lines, with the positions and sequences of the GTP-binding consensus elements shown in boxes. Below the lines are shown the amino acid substitutions made by site-directed mutagenesis. (B) The figure depicts how the NKXD consensus element may interact with the guanine ring, based on models of the G-domain of EF-Tu [9].

actually are transfected [6], this corresponds to greater than 25-fold overproduction in transfected cells.

DHFR synthesis was determined by SDS-PAGE and autoradiography (Fig. 3A) and its efficiency was calculated from DHFR mRNA levels determined by Northern blot analyses (Fig. 3B). The efficiency of DHFR synthesis in cells overproducing the various eIF2 mutant forms is reported in Table 1. When wildtype eIF2β is overproduced, DHFR synthesis is inhibited about 2.5-fold, an unexpected result that is seen consistently [12]. A possible explanation for this translational control phenomenon might be that overexpressed eIF2β interacts

Table 1
Quantitation of parameters from COS-1 cells overexpressing eIF2β (A) or eIF2γ (B) mutant forms

A. eIF2β

eIF2β construct	eIF2β accumulation[a]	DHFR mRNA level[b]	rate of DHFR synthesis[c]
pMT3	0.05	1.0	1.00
Wild type	1.00	1.2	0.40
N151Y	1.13	1.2	0.25
N151D	1.21	0.9	0.29

B. eIF2γ

eIF2γ construct	eIF2γ accumulation[a]	DHFR mRNA level[b]	rate of DHFR synthesis[c]
pMT3	0.06	1.0	1.00
Wild type 1.7 kb	0.95	0.9	0.79
N190D	0.92	1.1	0.09
N190Y	0.91	1.4	0.05
N190H	0.85	1.1	0.08

[a] The relative levels of accumulation of the protein forms were quantitated by densitometric scanning of Western immunoblots. The values were normalized to the overexpressed wild-type form, which was normalized to a value of 1.00.
[b] DHFR mRNA levels measured by phosphorimager quantitations from Northern blots. The values were corrected for the differences in the amount of RNA loaded on the gel by densitometric scans of the 18 and 28 S rRNA bands in a photographic negative of the ethidium bromide stained gels. Furthermore, these values were normalized to that from pMT3 alone.
[c] DHFR bands on the autoradiograms were quantitated by a phosphorimager and expressed as relative rate of DHFR synthesis. Value 1 was given to the cells transfected with pMT3 and 0, to the mock-transfected cells.

with the guanine nucleotide exchange factor eIF2B and interferes with the normal function of eIF2B by preventing its catalysis of GTP exchange on eIF2. When the N151Y or N151D mutant forms of eIF2β are overexpressed, DHFR synthesis is inhibited 3- to 4-fold. Were these mutant forms defective for GTP binding and therefore eIF2 function, a more subtantial inhibition of DHFR translation would be anticipated. In the case of eIF2γ, wildtype overproduction causes only slight inhibition of DHFR synthesis (ca. 20%). However, each of the three

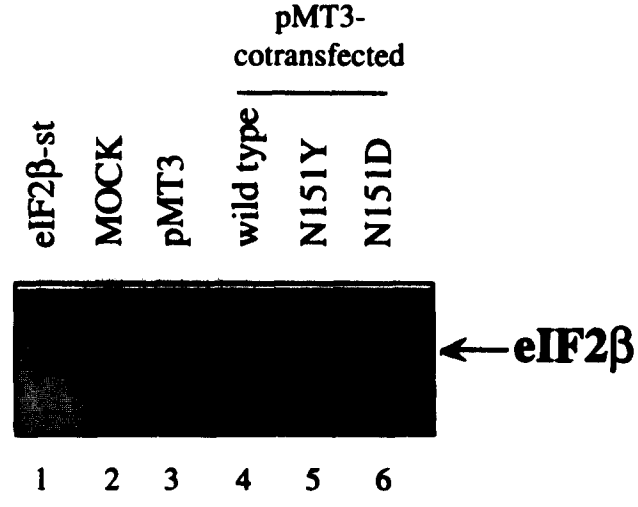

Fig. 2. Analysis of eIF2β accumulation in transfected COS-1 cells. COS-1 cells were cotransfected with pMT3 and pMT3–2β, labeled with [^{35}S]Met and lysed as described in Material and Methods. The overexpressed levels of eIF2β were compared to their normal cellular levels by 8% SDS-PAGE [7]. Cell lysates containing equal amounts of TCA-precipitable radioactivity were fractionated and electrotransfered to an Immobilon PVDF membrane (Milipore Corp.) using 10 mM CAPS pH 11 containing 10% methanol. The membrane was probed with rabbit anti-eIF2β serum [16] followed by alkaline phosphatase-conjugated goat anti-rabbit IgG. The membrane was developed with chromogenic substrates as described previously [5] and photographed. Lane 1, 300 ng purified eIF2; lane 2, mock-transfected cells; lane 3, cells transfected with pMT3 alone; lanes 4–6, cells cotransfected with pMT3 and pMT3–2β plasmids as indicated above the lanes.

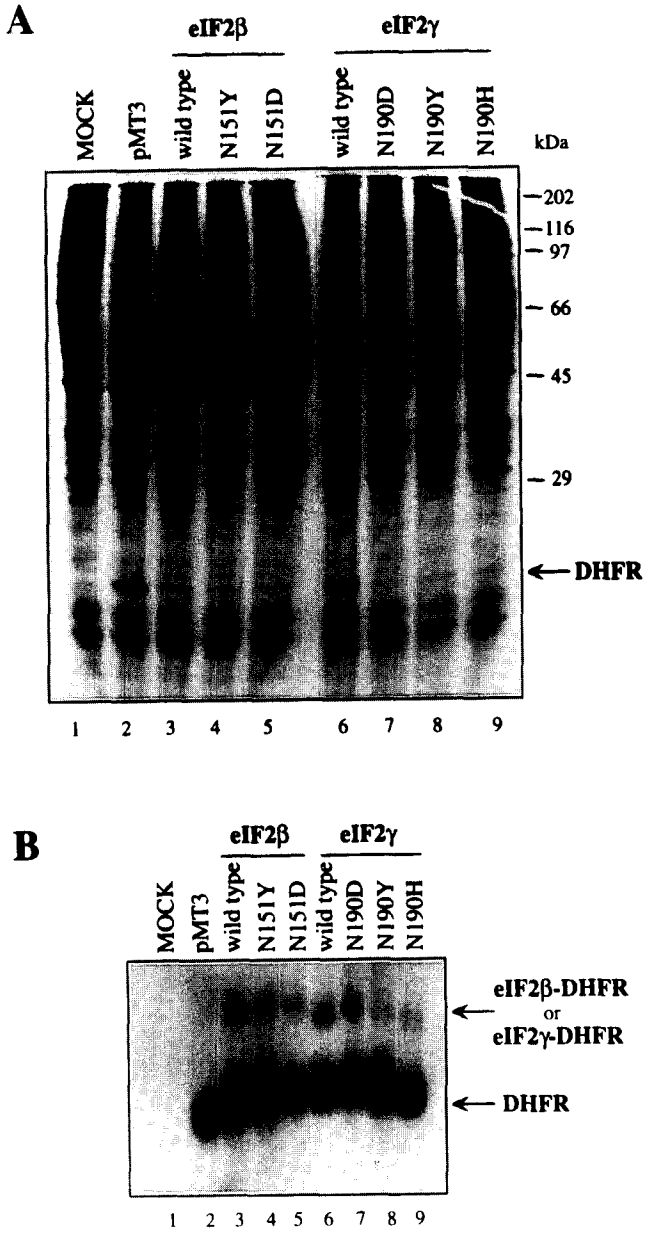

Fig. 3. Analysis of DHFR synthesis and mRNA levels. Transfected COS-1 cells were labeled with [^{35}S]Met and lysed as described in section 2. (A) The relative synthesis rates of DHFR were determined by analysis of the cell lysates containing equal amounts of radioactivity by 12% SDS-PAGE [7] followed by phosphorimager analysis (Bio-Rad, GS-250 molecular imager) of the gels. Values obtained were normalized to DHFR mRNA levels. Molecular weight markers are shown on the side and the DHFR band is labeled. Lane 1, mock transfected cells; lane 2, cells transfected with pMT3 alone; lanes 3–9, cells cotransfected with pMT3 and the pMT3-2β or pMT3-2γ plasmids as indicated in the figure. (B) Portions of the same cell lysates were analyzed for DHFR mRNA by Northern blot analysis. Total RNA from transiently transfected COS-1 cells was isolated as described [17], blotted onto a nylon membrane (Hybond-N, Amersham) and probed with a 0.7 kb *Eco*RI/*Sty*I fragment of the DHFR cDNA ^{32}P-labeled with a multiprime DNA labeling system (Amersham). The lanes correspond to those in panel A.

mutants, N190Y, N190D and N190H, cause greater than 10-fold inhibition of DHFR synthesis. The reduction of DHFR synthesis is not due to inhibition of transcription of its mRNA, as shown by Northern blot analysis (Fig. 3B). Comparable amounts of DHFR mRNA in the cells transfected with wildtype or the mutant forms were obtained.

4. Discussion

The results show that when the Asn residue is altered in the NKXD consensus element of the γ-subunit of eIF2, protein synthesis is strongly inhibited. In contrast, alterations of the corresponding Asn residue in the β-subunit cause little change in DHFR synthesis compared to the wildtype subunit. In both cases, the overproduced subunit is expected to exchange into the endogenous eIF2 complex, as demonstrated for eIF2α [13], thereby converting the bulk of the endogenous eIF2 into a mutant form. If the mutant eIF2 form cannot bind GTP, a strong inhibition of protein synthesis is expected. The results therefore support the view that GTP binding requires the NKID element in eIF2γ but does not involve the NKKD element in eIF2β. This conclusion is compromised somewhat by the fact that overproduction of wildtype eIF2β already causes some inhibition of protein synthesis, thereby masking to some extent the possible inhibition resulting from the amino acid substitutions in the eIF2β NKXD consensus element. However, since eIF2β overproduction is particularly strong and since the inhibition seen with the mutant form is only 3.5- to 4-fold, compared with greater than 10-fold for the corresponding eIF2γ mutant forms, the evidence supports the view that the eIF2β element plays no role in GTP binding.

Although eIF2β contains two of the three GTP binding consensus elements, these elements are separated by a distance far greater than is normally found in GTP-binding proteins [4]. Furthermore, the NKXD consensus element is entirely absent from yeast eIF2β [14], whereas all three consensus elements are present in yeast eIF2γ [15]. It therefore appears that GTP binds only to the γ-subunit in yeast. Since the yeast and human eIF2 proteins are highly conserved (50–72% sequence identity) and since the human eIF2β cDNA can substitute for the corresponding yeast gene in vivo (M. Kainuma, unpublished results), GTP binding likewise most likely occurs exclusively to the γ-subunit in mammalian cells. Unexplained, however, are the affinity crosslinking results with GTP analogs. A possible explanation is that eIF2β lies very close to the GTP binding site in the G-domain of eIF2γ. Structural analyses of the eIF2 complex are required to provide a full explanation of these perplexing results.

Acknowledgements: This work was support by NIH Grant GM22135 from the U.S.P.H.S.

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