A Specific Role for the Phosphorylation of Mammalian Acidic Ribosomal Protein P2*

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The acidic ribosomal proteins P1-P2 from rat liver were overproduced for the first time by expression of their cDNA in Escherichia coli. They were tested for their ability to reactivate inactive P1-P2-deficient core particles derived from 60 S ribosomal subunits treated with dimethylmaleic anhydride, in poly(U)-directed poly(Phe) synthesis. The recombinant P1-P2 were unable to reactivate these core particles although they could bind to them. When recombinant P1-P2 had been phosphorylated first with casein kinase II, they were as efficient in the reactivation process as P1-P2 extracted with ethanol/KCl from the 60 S subunits. Reconstitution experiments were carried out using all possible combinations of the two recombinant proteins phosphorylated or not. Reactivation of the core particles required the presence of both P1 and P2 with the latter in its phosphorylated form. These experiments reveal a distinct role for P1 and P2 in protein synthesis. Phosphorylated P2 produced a partial quenching of the intrinsic fluorescence of eukaryotic elongation factor 2, which was not observed with the unphosphorylated protein. This result demonstrates the existence of an interaction between phosphorylated P2 and eukaryotic elongation factor 2. P2 also quenched part of the intrinsic fluorescence of P1, due to the interaction between the two proteins.

The large subunit of eukaryotic ribosomes contains 12-kDa acidic P proteins, which seem more numerous in lower eukaryotes than in higher organisms (1). In these, two types of P proteins are found, designated as P1 and P2 (2, 3). They share similar properties with prokaryotic proteins L7–L12, which form a pentamer with protein L10, (L7/L12)_5-L10, constituting the large subunit of 50 S ribosomes (4). The eukaryotic P proteins, as their prokaryotic counterparts, seem to play an essential role in the interaction with elongation factors and in factor-dependent GTPase activity (5). However, some specific properties are observed with eukaryotic P proteins. First, these proteins exist on the ribosome as phosphorylated derivatives. They can be phosphorylated in vitro by either casein kinase II or by an endogenous ribosome-bound enzyme (6). Second, proteins P1-P2 present on the ribosome can exchange with a cytoplasmic pool of these unphosphorylated proteins (7). Phosphorylation of P1-P2, which appears to be necessary for ribosome activity, was originally suggested to be a requirement for the binding of these proteins to ribosomes (5), but recently this hypothesis has been challenged by new results obtained in yeast (8).

We have shown previously that active rat liver 60 S ribosomal subunits could be reconstituted from inactive core particles prepared with 2,3-dimethylmaleic anhydride (DMMA)1 (9), by adapting a method previously used with yeast ribosomes (10). Reactivation of the rat liver core particles was obtained not only with DMMA-split proteins containing several proteins including P1-P2 but also with a 50% ethanol, 0.08 M KCl extract containing P1-P2 exclusively. Dephosphorylation of P1-P2 with alkaline phosphatase completely inhibited the reactivation process (11). The poor ability of the ethanol/KCl core particles to be reactivated with this extract was shown to be related to a conformational alteration which destabilizes the 5 S RNA-protein complex (12).

Using this relatively simple in vitro reconstitution system, we here report results obtained by studying separately the role of P1 and P2 overproduced by cloning their cDNA in Escherichia coli. We have focused our work on the effects of phosphorylation of each of the two proteins, their mutual interaction, and the interaction of P2 with elongation factor eEF-2.

EXPERIMENTAL PROCEDURES

Materials—The primers used for the polymerase chain reaction were from Life Technologies, Inc. AmpliTaq DNA polymerase was from Perkin-Elmer. The pQE-30 plasmid and the Ni²⁺-nitrilotriacetic acid-agarose gel came from Qiagen. Restriction enzymes, T4 DNA ligase, and E. coli cells were from Promega. Ampicillin came from Boehringer Mannheim, Benzonase™ from Merck, and isopropyl-β-D-thiogalactopyranoside from MERCK. The pQE-30 plasmid and N-acetyltryosinamide were fromSigma. [γ-32P]ATP (specific activity, 11 Bq/pmol) and L-[14C]phenylalanine (specific activity, 20 Bq/pmol) were purchased from NEN Life Science Products. Preparation of rat liver eEF-2 (95% pure) and of 60 S ribosomal subunits by zonal centrifugation has already been described (13, 14). A monoclonal antibody prepared against recombinant P1 and reacting with P0-P1-P2 was a gift from Dr. Monier, Immunology Department, University Lyon 1, Lyon, France. Protein concentration was determined with the Coomassie Blue plus protein assay reagent kit from Pierce.

Construction of Expression Vectors—The plasmids pP1-13 and pP2-11 encoding, respectively, rat liver ribosomal proteins P1 and P2 were a gift from Dr. I. G. Wool (University of Chicago) and had been prepared as described (3). P1 cDNA was polymerase chain reaction-amplified from pP1-13 plasmid using a 5′ primer (5′ TAT GGA TCC ATG GTA TTA GGA ACC AAA GCC CAT 3′) and 3′ primer (5′ TAT AAG CTT TTA GGA ACC AAA GCC CAT 3′). P2 cDNA was polymerase chain reaction-amplified from pP2-11 plasmid using a 5′ primer (5′ TAT GGA TCC ATG GTA TTA GGA ACC AAA GCC CAT 3′) and a 3′ primer (5′ TAT AAG CTT TTA GGA ACC AAA GCC CAT 3′). The primers introduced BamHI and HindIII restriction sites, and the amplified cDNAs were digested by the endonucleases after gel purification and ligated into the corresponding sites of linearized pQE-30 (Qiagen) or pGEX-KX plasmids.

1 The abbreviations used are: DMMA, 2,3-dimethylmaleic anhydride; eEF-2, eukaryotic elongation factor 2; GST, glutathione S-transferase.

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The pQE-30 plasmid allowed the production of a protein carrying a 6-histidine tag at the N terminus. The pGEX-KT plasmid (a gift from Dr. D. Negre and Dr. J. C. Cortay, CNRS, Lyon, France), constructed according to Hakes and Dixon (15) is designed to generate an in-frame fusion protein composed of glutathione S-transferase (GST) and P1 or P2. pGEX-KT includes a thrombin cleavage site and an upstream gly-cine linker engineered between the GST and ribosomal protein sequences. The four recombinant plasmids were named pQE30-P1, pQE30-P2, pGEX-P1, and pGEX-P2. E. coli JM 109 cells (endA1, recA1, gyrA96, thi, hsdR17 (rk-, mk+), relA1, supE44, d(lac-proAB),ompT, trd36, proAB, lacYDAM15) were transformed with the fusion product and grown on agar plates supplemented with ampicillin (100 μg/ml). Correct recombinants were identified by multiple restriction digests.

Protein Overproduction—E. coli transformed with the recombinant plasmid were grown at 37 °C in LB medium (1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) at pH 7.5 containing 100 μg of ampicillin/ml, until the absorbance at 600 nm reached 0.7 unit. Expression of recombinant proteins was induced with 2 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C. Cells were harvested by centrifugation at 5,500 × g for 10 min at 4 °C and resuspended in 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 6 mM MgCl₂, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 100 units of Benzonase/ml. The cells were lysed using a SLM-Aminco French pressure cell press at 1,200 p.s.i. with a 40-ml capacity cell and centrifuged at 30,000 × g for 30 min.

Purification of the Recombinant Proteins—The acidic proteins expressed with pQE-30 plasmid were purified through a Ni²⁺-agarose affinity chromatography, according to the Qiagen instruction manual. The fusion proteins expressed with pGEX-KT plasmid were purified through glutathione-Sepharose 4B affinity chromatography, cleaved by thrombin (enzyme/substrate ratio, 1:160, by mass), and the released ribosomal proteins were purified after a subsequent glutathione-Sepha-rose 4B chromatography, according to the Pharmacia instruction manual. Recombinant proteins, purified in both systems, were then dialyzed against 50 mM Tris-HCl, pH 7.4, 20 mM KCl, 10% (w/v) glycerol, 4 mM 2-mercaptoethanol and frozen at -80 °C.

Reconstitution of Active Particles—DMMA core particles were prepared from 60 S ribosomal subunits as described previously (9). The selective extraction of proteins P1-P2 from 60 S subunits by treatment with ethanol and 0.08 M KCl has been also described (11). The reconstitution process was carried out using the DMMA core particles and either the ethanol extract (11, 12) or the recombinant proteins (phosphorylated or not). The amount of split proteins was three times in excess in the case of ethanol extract and two times in the case of the recombinant proteins.

Phosphorylation of Recombinant Proteins by Casein Kinase II—The casein kinase II was a gift from Dr. Chambaz (University of Grenoble, Grenoble, France). Recombinant proteins were phosphorylated by casein kinase II (enzyme/substrate, 1:800, by mass) at 37 °C for 30 min. These reactions were performed using a SLM-Aminco 8000C spectrofluorometer as described previously (17). The decrease of fluorescence intensity in the presence of increasing amounts of P2 phosphorylated or P1 fluorescence was deduced from the decrease of the fluorescence intensity in the presence of increasing amounts of P2 phosphorylated or not. Fluorescence was corrected for dilution. Correction for inner filter effect of P2 was made using N-acetyltryptophanamide as a standard.

RESULTS

Overexpression and Purification of Ribosomal Proteins P1 and P2—Fig. 1 shows that the ribosomal protein P2 with the 6-histidine tag at the N terminus (A) as well as the GST-P2 fusion protein of M₂, 40,000 (B) were highly overexpressed upon isopropyl-β-D-thiogalactopyranoside induction, as a soluble form in the supernatant. Using the first system, affinity chromatography on a Ni²⁺-agarose column was a particularly effi-cient purification procedure in which the recombinant riboso-mal proteins were selectively bound and then subsequently eluted with 150 mM imidazole. In the second system of expres-sion, an efficient purification of the fusion protein was obtained by affinity chromatography through a glutathione-Sepharose 4B column. This fusion protein was very sensitive to thrombin cleavage, generating isolated P2 and GST without any appar-ent secondary cleavage. P2 was then purified using a second glutathione-Sepharose 4B column, as the unbound fraction, while GST and the small amount of uncleaved fusion protein were retained. The yield was 100 mg of protein, approximately 99% pure, per liter of E. coli culture using the first system and 10 mg using the second one. Similar results were obtained for ribosomal protein P1 overexpressed in both systems, but P1 released after cleavage of the GST-P1 fusion protein was insoluble.

The M₆ of the two recombinant proteins estimated by their migration in SDS-polyacrylamide gel electrophoresis was signifi-cantly higher than the theoretical values of 11,490 and 11,684 for P1 and P2, respectively. A similar difference between observed and expected values has been already observed using native P1 and P2 (2). In our case, it is also necessary to take into account the supplementary N-terminal residues. To verify the correct processing of the recombinant proteins, these were electroblotted onto a polyvinylidene difluoride membrane and submitted to N-terminal sequencing by automatic Edman degradation using a 473 A liquid sequencer (Applied Biosys-tem). The correct N-terminal residues of both proteins were found, preceded by GS in the case of the protein obtained from GST-P2 and by MRGS/H₄K₃OS in the case of the recombinant proteins with the 6-histidine tag.

Reconstitution of Active 60 S Ribosomal Subunits Using Phosphorylated Recombinant P1-P2—Core particles were prepared from 60 S ribosomal subunits treated with DMMA as described previously (9). The absence of P1-P2 proteins from these particles was confirmed using a monoclonal antibody prepared against recombinant P1 and which reacted with P0, P1, and P2 in the 60 S subunits (Fig. 2A). The core particles contained P0, but not P1 nor P2 (Fig. 2B), and exhibited only 5% of the original activity of the 60 S subunits in poly(U)-directed polyphenylalanine synthesis. After incubation with recombinant P1-P2, the activity of the core particles was still very low, as compared with that measured after addition of an ethanol extract of 60 S subunits containing native P1-P2 (14% instead of 82%) (Table I). Recombinant P1-P2 were then phosphorylated with casein kinase II and ATP. As shown in Fig. 3, there was no remaining unphosphorylated form after incubation of
P1 and P2 with the kinase under the phosphorylation conditions used. The extent of core particle reactivation observed (85%), after addition of the two recombinant proteins previously phosphorylated, was identical to that observed with native P1-P2. Addition of phosphorylated P1 and unphosphorylated P2 produced a very small reactivation (10%). Interestingly, addition of phosphorylated P2 and unphosphorylated P1 gave a much better reactivation (63%). The same results were obtained using either P2 with the 6-histidine tag or P2 overexpressed with the 6-histidine tag at the N terminus were added to DMMA core particles as indicated. The 100% value corresponds to 11,350 cpm (i.e. 16 pmol) of [3H]polyphenylalanine incorporated. Results are the mean values found in three different reconstitution experiments.

| Preparation | Addition       | Poly(Phe) synthesis |
|-------------|----------------|--------------------|
| 60 S subunits | Ethanol extract | 82                 |
| 60 S subunits | P1 + P2        | 5                  |
| 60 S subunits | Phosphorylated (P1 + P2) | 85               |
| 60 S subunits | P1 + Phosphorylated P2 | 10               |
| 60 S subunits | Phosphorylated P1 | 63                |
| 60 S subunits | Phosphorylated P2 | 8                 |

To see whether or not unphosphorylated P1-P2 were able to bind to the core particles, reconstituted subunits were phosphorylated with casein kinase II and [γ-32P]ATP and their protein content analyzed by gel electrophoresis and autoradiographed (Fig. 4). As expected from the preceding results, no residual P1-P2 was labeled in the core particles treated in this way (lane 1). On the other hand, subunits reconstituted from these core particles and unphosphorylated P1-P2 contained the two labeled proteins (lane 2). Only P1 was labeled in subunits reconstituted using phosphorylated P1 with P2 previously phosphorylated with cold ATP (lane 3). In the reverse situation (unphosphorylated P2 with P1 phosphorylated with cold ATP), only P2 was labeled (lane 4).

FIG. 2. P protein content of 60 S subunits and DMMA core particles. 63 μg of 60 S subunit proteins (A) or 41 μg of core particle proteins (B) were separated on a 15% SDS-polyacrylamide gel (lane 2) and transferred to a nitrocellulose sheet (lane 3). Immunostaining was carried out using a monoclonal antibody and anti-immunoglobulins as described previously (16). Lane 1, molecular size markers.

FIG. 3. Phosphorylation of recombinant proteins P1-P2. 6 μg of phosphorylated proteins (lanes 1 and 3) and unphosphorylated proteins (lanes 2 and 4) were analyzed by isoelectric focusing using a 4–7 pH gradient from bottom to top in a 6% polyacrylamide gel containing 9 M urea.

FIG. 4. Phosphorylation of reconstituted subunits. DMMA core particles and reconstituted subunits were phosphorylated using casein kinase II and [γ-32P]ATP, and their protein content was analyzed on a 15% SDS-polyacrylamide gel, which was autoradiographed for 6 h. Lane 1, DMMA core particles; lane 2, subunits reconstituted from core particles + recombinant P1-P2; lane 3, core particles + P1 + P2 previously phosphorylated with cold ATP; lane 4, core particles + P2 + P1 previously phosphorylated with cold ATP.

Effect of Ribosomal Protein P2 on eEF-2 and P1 Intrinsic Fluorescence—The addition of phosphorylated P2 to eEF-2 induced a partial quenching of the intrinsic fluorescence of eEF-2 (Fig. 5, open circles). eEF-2 contains 7 Trp residues and P2 none. A maximal quenching of 13% of eEF-2 intrinsic fluorescence was observed with a slight blue shift (from 332 to 328 nm). The concentration of phosphorylated P2, which produced 50% of the maximal eEF-2 fluorescence quenching, was 100 nM. On the other hand, addition of the unphosphorylated P2 had no effect at the same concentrations (closed circles). These results demonstrate the existence of an interaction between eEF-2 and P2, but only when the latter protein is phosphorylated. Using the same technique to study the interaction between P1 and eEF-2 was complicated by the fact that the presence of a Trp residue in P1 required the use of correction factors. In another experiment, we found that P2 quenched 20% of the intrinsic fluorescence of P1 when both proteins were added in an equimolar ratio (result non shown).

DISCUSSION

By expressing the cDNA of mammalian acidic ribosomal proteins P1 and P2 in an E. coli system, it was possible to obtain these proteins pure in large amounts and therefore to study the function and the role of the phosphorylation of each protein. Active 60 S subunits could be reconstituted by incubation of inactive DMMA core particles with the recombinant proteins previously phosphorylated. The requirement for phosphorylation to obtain active material was not surprising, since this result has also been obtained in reconstitution experiments using P1 and P2 extracted from the ribosomes (5) or the 60 S subunits (11). More unexpected was the finding of a dissymmetry between the effects of phosphorylated P1 and P2:
phosphorylated P2 with unphosphorylated P1 could reactivate the core particles, whereas phosphorylated P1 with unphosphorylated P2 were almost ineffective. The phosphorylation reaction was carried out with casein kinase II. It has been shown that the two residues, which are phosphorylated by this enzyme as well as by an endogenous ribosome-bound kinase, are the two serine located in the common C-terminal peptide near the C terminus (6). From the results of our experiments, it is clear that the phosphorylation of P1-P2 is not required for their binding to the core particles, since the unphosphorylated proteins can be incorporated and then phosphorylated with \( \gamma^{\text{32P}} \text{ATP} \). The presence in the core particles of P0, which is assumed to be required for the binding of P1-P2 (18), could be demonstrated by using a monoclonal antibody. This antibody had been obtained using recombinant P1 as antigen, and since it recognized the three proteins P0-P1-P2, it was directed most likely against an epitope located in the C-terminal peptide common to the three proteins. The major part of P0 was probably phosphorylated, since this protein was not visible in the autoradiogram of the core particles incubated with the kinase and \( \gamma^{32P} \text{ATP} \) (Fig. 4). In fact, it appeared weakly labeled when the exposure time was increased (not shown). If the role of P1-P2 phosphorylation is not to allow the binding of these proteins to the core particles, the questions of why it is required for the reactivation process and why P2 phosphorylation appears to have a specific role remain. The experiment illustrated by Fig. 5 could give at least part of the answer. It had been shown previously that elongation factor eEF-2 could be cross-linked with protein P2, among other ribosomal proteins (19). Our results obtained from intrinsic fluorescence measurements demonstrate the existence of an interaction of eEF-2 with phosphorylated P2. On the other hand, no interaction between eEF-2 and unphosphorylated P2 was observed using this technique. It will be interesting now to identify the amino acid residues of P2, which are involved in this interaction by site-directed mutagenesis and to precise the exact role of P2 phosphorylation in the eEF-2-dependent step of elongation. Interaction of P2 with P1 was also detected using the same technique, and the amino acid residues involved should also be identified.

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