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Association of the Heme-Controlled eIF-2α Kinase with Spectrin-Derived Peptides

G. Kramer, W. Kudlicki, S. Fullilove, and B. Hardesty

Translational control of mammalian protein synthesis frequently occurs at the level of peptide initiation. One control system in particular has been intensively studied during the last decade. It involves phosphorylation and dephosphorylation of the smallest subunit (α-subunit) of initiation factor 2, eIF-2. Two different substrate-specific protein kinases are recognized that can carry out this phosphorylation and thereby cause inhibition of protein synthesis. Both occur in inactive form in mammalian cells [1, 2]. One of the kinases is induced by interferon and activated by double-stranded RNA. The other is activated under conditions of heme deficiency and is known as the heme-controlled repressor (HCR) kinase. Our attention has been focused on the mechanism by which the latter enzyme is activated and regulated in rabbit reticulocytes and extracts of these cells. Although details of the mechanism are not known, we have evidence that spectrin and peptides derived from it by proteolysis are involved in its regulation [3]. Highly purified preparations of the kinase contain a prominent peptide of M_r 90000, a phosphopeptide of M_r 100000, and minor peptides of higher molecular weight. The M_r 90000 peptide was shown to react with monoclonal antibodies against spectrin, thus indicating that these peptides are structurally related. Other results led to the hypothesis that the M_r 90000 peptide is derived from the C-terminal end of β-spectrin by proteolysis [3]. Here we describe experiments extending our studies on the relationship of the M_r 90000 peptide to spectrin and the modulation of the HCR kinase activity by this peptide.

Monoclonal antibodies were raised against the highly purified M_r 90000 peptide. This peptide was separated from HCR activity and obtained in apparently homogeneous form by polyacrylamide gel electrophoresis run under nondenaturing conditions. The preparation had no HCR kinase activity. The purified peptide was injected directly into the spleen of a Balb/c mouse. After 4 days, the spleen was excised and the spleen cells were fused with myeloma cells. The resulting hybridomas were grown and assayed in an enzyme-linked immunosorbent assay (ELISA) [4]. Positive hybridomas were cloned; individual clones were grown and eventually used to produce ascites. Antibodies (IgM class for those used here) were partially purified by ammonium sulfate precipitation followed by chromatography on Sephacryl S300.

In Fig. 1, the antibodies raised against the M_r 90000 peptide are characterized. Peptides from an eIF-2α kinase preparation were separated on a denaturing polyacrylamide gel. Part of the gel was stained (Fig. 1 A, track 1). The other part was used for electrophoretic transfer of the peptides onto nitrocellulose which was then probed with the antibodies (Fig. 1 B). Subsequently, an antimouse second antibody linked to peroxidase was added. The reaction of the monoclonal antibodies with specific pep-

Clayton Foundation Biochemical Institute, Department of Chemistry, The University of Texas at Austin, Austin, Texas 78712, USA
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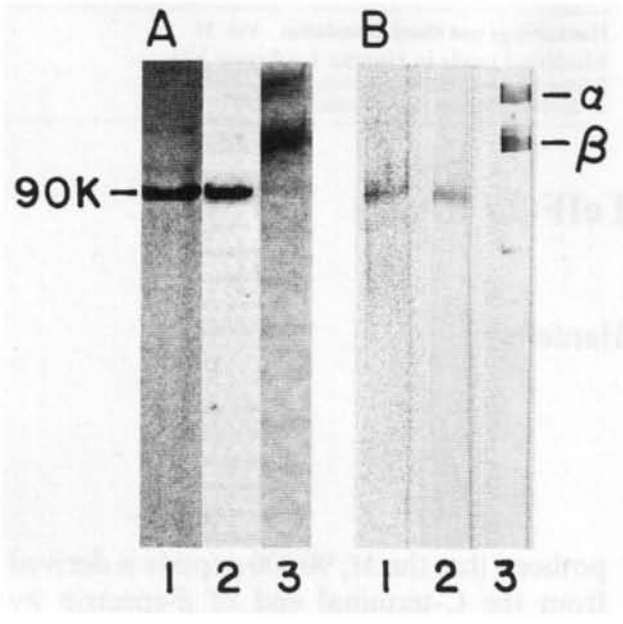

Fig. 1 A, B. Reaction of monoclonal antibodies with peptides of the eIF-2α kinase preparation and spectrin. Highly purified eIF-2α kinase (*track* 1), the isolated M_r 90000 peptide (*track* 2), and spectrin (*track* 3) isolated from rabbit erythrocytes according to Ungewickell and Gratzer [5] were electrophoresed on 15% (*tracks* 1 and 2) or 7½% (*track* 3) polyacrylamide gels in sodium dodecylsulfate. **A** The separated peptides stained with silver (*track* 1) or Coomassie Blue (*tracks* 2 and 3). **B** Peptides stained with Biorad horseradish peroxidase substrate after the ELISA [4], using the monoclonal antibodies raised against the M_r 90000 peptide

tides was visualized by applying a colored substrate for the peroxidase [3, 4], with the results shown in track 1. The M_r 90000 peptide was visible after this ELISA procedure. For comparison, the purified M_r 90000 peptide (track 2) and spectrin (track 3) were used as the antigen. There is evidence in the literature that monoclonal antibodies recognize both subunits of spectrin [6], and homology between both subunits has been demonstrated [7]. No reaction is seen when either the first or the second antibody is omitted, or when regulin [4] is substituted for spectrin. The data presented in Fig. 1 thus provide further evidence that the M_r 90000 peptide of the eIF-2α kinase is related to spectrin. Interestingly, the same antibodies described above also recognize the prominent M_r 120000 peptide found in a highly purified eIF-2α phosphatase fraction. This peptide has been detected by monoclonal antispectrin antibodies (Hardesty et al., this volume).

The monoclonal antibodies against the M_r 90000 peptide characterized in Fig. 1 affect the activity of the eIF-2α kinase, thus indicating a regulatory function of the spectrin-derived peptide. Phosphorylation of eIF-2α is analyzed by polyacrylamide gel electrophoresis in sodium dodecylsulfate. An autoradiogram is prepared from the dried gel, the part of the gel containing the stained α-subunit is then cut, and its radioac-

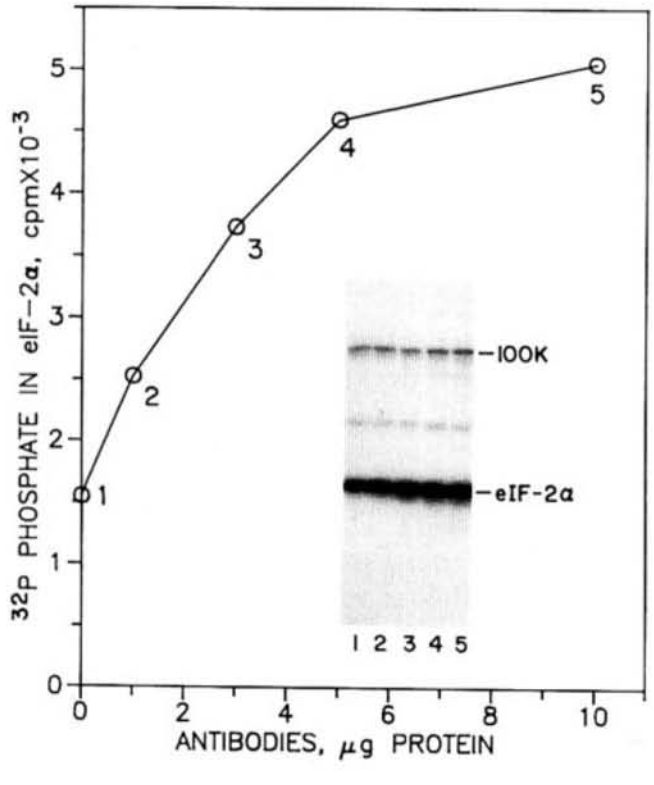

Fig. 2. Monoclonal antibodies against the M_r 90000 peptide stimulate eIF-2α kinase activity. The enzyme (cf. 3; about 0.2 μg of protein) was preincubated with the indicated amount of the anti-M_r 90000 antibodies for 30 min on ice before the phosphorylation reaction was carried out in the presence of about 5 μg eIF-2 and 0.1 m*M* [γ^{-32}P]ATP (about 2 Ci/mol). The reaction mixtures were analyzed on 15% polyacrylamide gels in sodium dodecylsulfate. The gel was stained with Coomassie Blue and an autoradiogram was prepared. The part of the gel corresponding to eIF-2α was then cut and its radioactivity determined by scintillation counting. The results from the incubations in the absence or presence of the antibodies are given. The *insert* represents the autoradiogram

tivity determined. Results thus obtained from the experiment with monoclonal antibodies are presented in Fig. 2. Preincubation of the eIF-2α kinase preparation with the anti-M_r 90 000 peptide antibodies causes an increase in enzyme activity. No such increase is seen when nonimmune mouse IgG or monoclonal antiregulin antibodies [4] are used (data not shown). These results appear to indicate that the HCR kinase is associated with a peptide derived from the β-subunit of spectrin which plays an important role in regulation of its enzymatic activity. Further implications of these findings are discussed in the chapter by Hardesty et al. in this volume.

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