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Involvement of the Membrane Skeleton in the Regulation of the cAMP-Independent Protein Kinase and a Protein Phosphatase that Control Protein Synthesis

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A. Introduction

The ultimate targets of transformational changes in cells are the sites in the nucleus and cytoplasm that affect transcriptional and translational control of protein synthesis. Products of a number of oncogenes appear to be analogues of proteins involved in the steps of transmembrane signalling (hormones or growth factors, receptors, G-type regulatory proteins, and tyrosine kinases), and some insight into the specific mechanism is emerging (for review see [1]). However, little is known of the mechanism by which a signal is transmitted from the inner surface of the plasma membrane to specific targets in the nucleus and cytoplasm. In at least some cases, changes in the activity of specific cyclic adenosine monophosphate (cAMP)-independent protein kinases and possibly phosphoprotein phosphatases appear to be involved. The counterpoised activities of these enzymes determine the phosphorylation level of their protein substrates.

Eukaryotic peptide initiation factor 2 (eIF-2) can be phosphorylated in its smallest subunit – α-subunit – by either of two different substrate-specific, cAMP-independent kinases (for review see [2, 3]). This phosphorylation blocks the release of guanosine diphosphate (GDP) from eIF-2 by the GDP exchange factor [4, 5] and thereby prevents eIF-2 from functioning in peptide initiation. One kinase is induced by interferon and is activated by double-stranded RNA. It also appears to be involved in adenovirus-mediated control of host cell protein synthesis [6]. The other kinase is activated in reticulocytes under conditions of heme deficiency and is known as the heme-controlled repressor (HCR) (cf. [2, 3]). An eIF-2α kinase activated during heat shock of HeLa cells [7] was shown to be inhibited by antibodies against reticulocyte HCR [8]. Both kinases appear to phosphorylate the same serine residue(s) [9] in the N-terminal segment of eIF-2α [10, 11]. Here we describe the effect of the β-subunit of spectrin and peptides derived from it on the activity of the HCR protein kinase and protein phosphatase from rabbit reticulocytes that phosphorylate and dephosphorylate eIF-2α.

The isolation and characterization of these enzymes have proven to be particularly difficult. Both enzymes appear to be physically heterogeneous in size and may exist in inactive form in fresh cell extracts. Problems arise in the accurate quantitation of the enzymes (active and inactive forms) that are present in different cell fractions, particularly those containing membranes and the membrane skeleton. Although the details remain unclear, some insight into the basis for the physical heterogeneity and mechanism by which the enzymes are regulated is beginning to emerge.

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B. HCR Kinase

Although heterogeneous, as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS), our highly purified preparations of the kinase (purified >5000-fold on the basis of activity) contain a prominent M_r 90000 peptide (cf. Fig. 1) that does not have enzymatic activity by itself [12, 13]. Repeated attempts to isolate monoclonal hybridomas that would produce antibodies against the kinase resulted in antibodies that recognized this M_r 90000 peptide [13]. As shown in Fig. 1, these antibodies also react with spectrin, the major protein component of the erythroid membrane skeleton. Without exception, of the considerable number of monoclonal antibody isolates we have tested, all those that recognize the M_r 90000 peptide also recognize β-spectrin (M_r 220000). Most of the monoclonal antibodies also recognize the M_r 240000 α-subunit of spectrin, as shown in Fig. 1. There is considerable sequence homology between the two spectrin subunits [16].

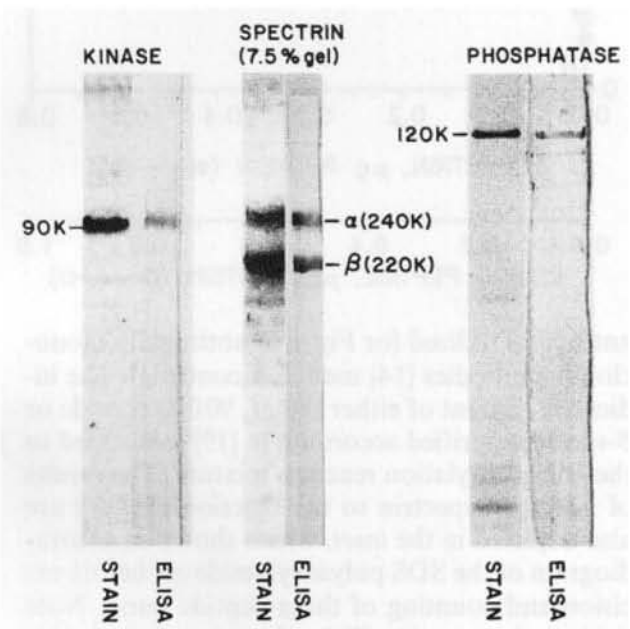

Fig. 1. Monoclonal antispectrin antibodies recognize both the M_r 90000 peptide of the eIF-2α kinase and the M_r 120000 peptide of the phosphoprotein phosphatase. Highly purified eIF-2α kinase or phosphatase was electrophoresed on 15% polyacrylamide gels in SDS. Part of the gel was stained with silver (ICN Rapid Ag Stain), the other part being electrophoretically transferred onto nitrocellulose which was then probed with the monoclonal antispectrin antibodies in an enzyme-linked immunosorbent assay (ELISA) (cf [14]). The antibodies used were of the IgM class and had been purified from ascites fluid by 0%–50% ammonium sulfate precipitation followed by chromatography on Sephacryl S300. Spectrin, isolated from rabbit erythrocytes according to [15], is shown for comparison. The spectrin subunits were separated on a 7½% polyacrylamide gel in SDS and then treated as described above, except that the first track is from a Coomassie-stained gel. 90K, 120K, 220K, and 240K denote the respective peptides of that size

Similarity between the M_r 90000 peptide and β-spectrin is also indicated by phosphorylation with two protein kinases. The β-subunit of spectrin contains one threonine and three serine residues within a M_r 10000 region at the C-terminal end that can be phosphorylated [17]. In vitro, the catalytic subunit of the cAMP-dependent protein kinase and the cAMP-independent casein kinase II phosphorylate the β-subunit of spectrin. Both of these protein kinases also phosphorylate the M_r 90000 peptide [13]. Furthermore, a phosphorylated M_r 90000 peptide can be derived in vitro by proteolysis from phosphorylated β-spectrin [13]. Considered together, these results indicate that the M_r 90000 peptide is structurally related to a segment at the C-terminal end of β-spectrin and probably derived from it by proteolysis.

At the stage of purification of the kinase used for Fig. 1, the M_r 90000 peptide is the most abundant component in the preparation. However, traces of other spectrin peptides are present, and we have isolated fractions with high HCR kinase activity in which the most abundant component is one of several higher molecular weight spectrin fragments. Some of these fractions contain no M_r 90000 peptide. β-spectrin itself and the M_r 90000 fragment have been isolated and have no detectable kinase or phosphatase activity. The catalytic subunit of the kinase appears to be a M_r 95000 peptide that is associated with the spectrin fragments. Attempts to isolate active kinase free of all spectrin peptides have not been successful.

The enzymatic activity of the HCR kinase is markedly increased either by the antispectrin monoclonal antibodies that recognize the M_r 90000 peptide or by the M_r 90000 peptide itself, β-spectrin, and some of the spectrin peptides of intermediate size. The

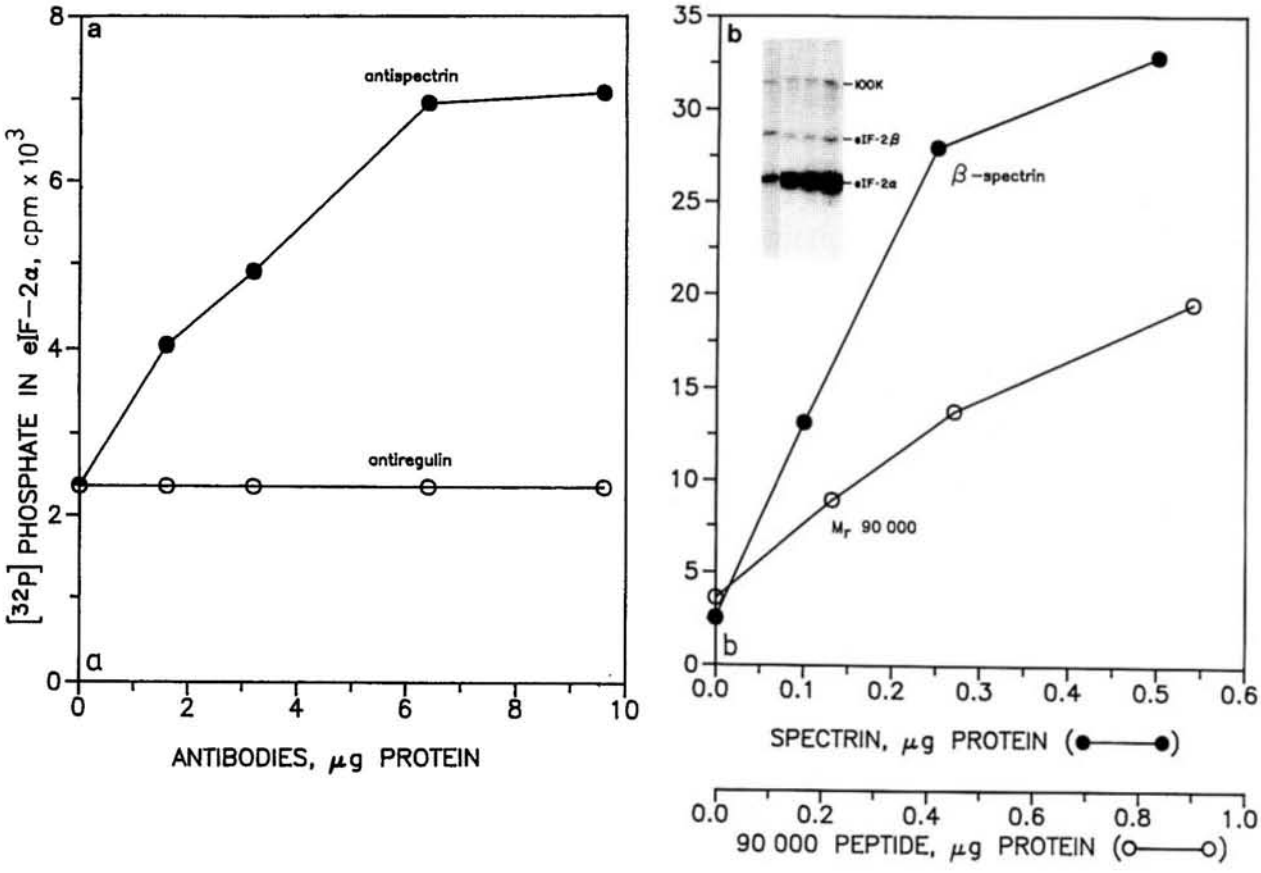

Fig. 2 a, b. Stimulation of HCR eIF-2α kinase activity by antispectrin monoclonal antibodies or by β-spectrin and M_r 90 000 peptide. About 0.2 μg of protein from the HCR kinase preparation was incubated with 5 μg of purified eIF-2 and 0.1 m*M* [γ^{32}P] adenosine triphosphate (2 Ci/mmol) under the conditions previously described [13]. The samples were electrophoresed in SDS on 15% polyacrylamide gels; the eIF-2α band was then excised and its radioactivity determined by liquid scintillation counting. **a** The HCR kinase preparation was preincubated for 30 min on ice with the indicated amount of the antispectrin monoclonal antibodies utilized for Fig. 1 or antiregulin monoclonal antibodies [14] used as a control. **b** The indicated amount of either the M_r 90 000 peptide or β-spectrin purified according to [19] was added to the phosphorylation reaction mixture. The results of adding β-spectrin to the reaction mixture are also depicted in the *inset*, which shows an autoradiogram of the SDS polyacrylamide gel before excision and counting of the α-peptide band. Note the large increase in eIF-2α phosphorylation, with little or no effect on the phosphorylation of eIF-2β or the M_r 100 000 phosphopeptide of the HCR kinase preparation

effect on eIF-2α phosphorylation of the antibodies used for Fig. 1 is shown in Fig. 2 a. The effect of the M_r 90 000 peptide and β-spectrin on enzymatic activity is shown in Fig. 2 b. The level of eIF-2α phosphorylation was determined by excising and counting the α-subunit from SDS polyacrylamide gels. The inset in Fig. 2 b shows an autoradiogram of such a gel. The mechanism by which kinase activity is increased is not clear.

C. Phosphoprotein Phosphatase

A Mn^{2+}-dependent protein phosphatase with high activity for dephosphorylation of eIF-2α was isolated from rabbit reticulocytes as part of our effort to characterize the components responsible for translational regulation of protein synthesis. The enzyme has many of the physical characteristics that are described above for the HCR kinase, ex-

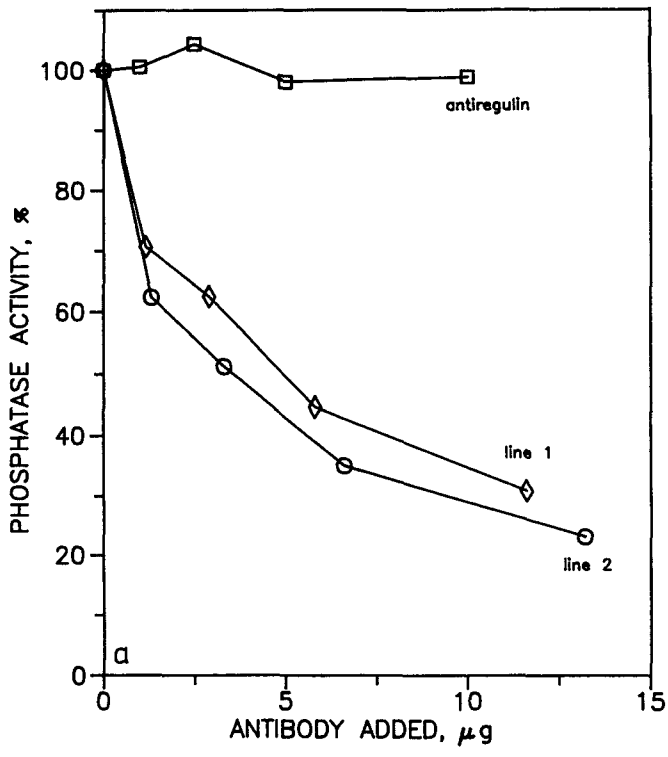

Fig. 3. Inhibition of protein phosphatase activity by antispectrin monoclonal antibodies. The phosphatase preparation (0.1 μg of protein) was incubated with [^{32}P]eIF-2(α-P) (about 46 pmol of [^{32}P]phosphate, 1 Ci/mmol). The dephosphorylation reaction was carried out in the presence of 0.25 m*M* Mn^{2+} and released phosphate determined as described previously [18]. 100 percent of enzyme activity equals the release of 5.0 pmol of [^{32}P]phosphate from the substrate in a 10-min dephosphorylation reaction. The enzyme preparation was preincubated for 1 h on ice with the indicated amount of monoclonal antibodies from three hybridoma lines. *Line 1* is that used in Fig. 1. *Line 2* is the monoclonal hybridoma isolate described by Kramer et al., this volume. Antiregulin monoclonal antibodies [14] were used as a control

cept that the most abundant component of highly purified preparations is a M_r 120000 peptide (Fig. 1). Minor peptides of lower molecular weight are visible in the silver-stained track. Most monoclonal antibody isolates that react with β-spectrin and the M_r 90000 peptide of the HCR kinase preparation also recognize the M_r 120000 peptide, as exemplified in Fig. 1. However, in contrast to their effect on the HCR kinase, some of these monoclonal antibody isolates inhibit the enzymatic activity of the phosphatase, as indicated by the data in Fig. 3. Monoclonal antibodies from hybridoma line 1 were those used for the data in Figs. 1 and 2. Monoclonal antibodies against regulin, a protein with physical properties somewhat similar to those of spectrin [14], have no effect on this protein phosphatase.

D. Discussion

The results described above indicate that both the HCR kinase and the phosphoprotein phosphatase interact with β-spectrin and peptides probably derived by proteolysis from its C-terminal region. The enzymatic activity of the kinase is increased by the interaction, whereas the activity of the phosphatase is decreased. Intact $(\alpha, \beta)_2$ spectrin has no effect on the enzymatic activity of either the phosphatase or the kinase. It appears that proteolytic generation of the β-spectrin peptides in vivo would increase eIF-2α phosphorylation and reduce protein synthesis. The β-subunit itself has been shown to be a potent inhibitor of protein synthesis in the reticulocyte lysate system [19]. Association with β-spectrin and peptides derived from it appears to account for some of the physical properties of both the HCR kinase and the phosphoprotein phosphatase. In purified preparations both appear to be highly elongated structures, apparently reflecting their association with the spectrin peptides. In both cases the axial ratio was calculated from the Stokes' radius, measured by gel filtration chromatography, and from the sedimentation coefficient, measured by glycerol gradient centrifugation.

Spectrin [20, 21] is an unusually protease-sensitive 200 nm rod. The α- and β- subunits are aligned side to side to form heterodimers which associate head to head to form a tetrameric structure that is the major component of the two-dimensional network which is the membrane skeleton of mammalian erythrocytes. It is characteristic of erythroid cells; however, proteins with extensive structural homology to the β-subunit of spectrin occur in nonerythroid cells [22, 23]. Brain tissue contains an α, β-dimer [24] that appears to be structurally related to erythroid spectrin.

There is some indication that the C-terminal portion of the β-spectrin subunit may be related to certain other M_r 90000 peptides

found to be a component of steroid [25, 26] and glucocorticoid [27, 28] receptors, of the pp60^{src} complex [29] and the M_r 84000–90000 heat shock protein [30]. We have observed that monoclonal antibodies (obtained from Dr. David Toft, Mayo Clinic, Rocheser, Minnesota [26]) which recognize the M_r 90000 peptide of the progesterone receptor also recognize the β-subunit of spectrin (unpublished data). Toft and coworkers have demonstrated cross-reactivity of the progesterone-receptor-associated M_r 90000 peptide and the peptide of the same size that can form a cytosolic complex with pp60^{src} [31]. Also, the steroid receptor M_r 90000 peptide and a heat shock protein (M_r 90000) were shown to be very similar, if not identical, by peptide mapping [32].

The effects of heat shock and stress on protein synthesis in mammalian cells have been studied intensively. Heat shock causes a dramatic shut-off of protein synthesis followed by accumulation of several characteristic heat shock proteins, including the M_r 90000 peptide. The heat shock response has been attributed to an effect on the cytoskeleton [33, 34]. Alterations in the cytoskeleton organization that lead to release of β-spectrin or peptides derived from it may be involved in the inhibition of protein synthesis caused by heat shock. Several recent reports indicate that some oncogenes may affect the cytoskeleton. Aberrant cytoskeletal organization was observed in B-cell leukemia and hairy-cell leukemia [35]. The v-*fgr* oncogene of the Gardner-Rasheed strain of feline sarcoma virus encodes a 128-amino acid peptide from γ-actin [36], and the *onc D* gene from human colon carcinoma appears to code for a protein with a 221-amino acid sequence from nonmuscle tropomyosin [37].

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