Phosphorylated and other modified forms of eukaryotic ribosomal protein S3 analysed by two-dimensional gel electrophoresis

David P. LEADER

Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

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Proteins were isolated from the 40S ribosomal subunits of baby-hamster kidney fibroblasts and subjected to two-dimensional gel electrophoresis. When the cells were pretreated with cyclic AMP or 2-deoxyglucose a more basic derivative of ribosomal protein S3 or S3a was often observed, apparently similar to that previously reported to occur early in liver regeneration. This derivative was not a dephosphorylated form of protein S3, which protein does not appear to be phosphorylated in normal cells; nor did it correspond to the proteolytic fragment, S3b. It appears to be an oxidation product of protein S3 or S3a, as it can be eliminated by thorough reduction of the ribosomal protein before electrophoresis. In contrast with previous results with Krebs II ascites cells, starvation of baby-hamster kidney fibroblasts for glucose did not cause extensive phosphorylation of ribosomal protein S3.

Interest in the physiological modification of eukaryotic ribosomal proteins has centred mainly on phosphorylation [see Leader (1980) for review]. Ribosomal protein S6 [nomenclature according to Sherton & Wool (1972) and McConkey *et al.* (1979)] has received particular attention, as the extent of its phosphorylation can be changed by a variety of stimuli, including cyclic nucleotides (Cawthon *et al.*, 1974; Gressner & Wool, 1976), alteration of cellular growth conditions (Leader *et al.*, 1976; Lastick *et al.*, 1977; Haselbacher *et al.*, 1979) and partial hepatectomy (Gressner & Wool, 1974). Anderson *et al.* (1975) have reported that another ribosomal protein, S3 (S2 in their nomenclature), is modified shortly after partial hepatectomy and, solely on the basis of the observed change in the electrophoretic mobility of the protein during two-dimensional gel electrophoresis, suggested dephosphorylation as a possible cause.

In our own subsequent studies of the incorporation of [32 P]P_i into the ribosomal proteins of Krebs II ascites cells we did, in fact, find that protein S3 is phosphorylated in certain circumstances (Leader & Coia, 1978a). However, in ascites cells the phosphorylation of protein S3 could only be detected under very abnormal conditions, such as glucose deprivation, whereas in rat liver this protein would have to be phosphorylated under normal conditions for it to become dephosphorylated after partial hepatectomy. As there is some evidence that protein S3 may be of functional importance in the

initiation of protein biosynthesis (Noll *et al.*, 1978), it seemed desirable to try to investigate this question further. The present paper reports data that suggest that ribosomal protein S3 is not normally phosphorylated in animal cells, and that an alteration in the electrophoretic mobility of protein S3, similar to that observed by Anderson *et al.* (1975), can occur through the oxidation of the thiol groups of cysteine residues in the protein.

Experimental

BHK21/C13 (baby-hamster kidney) cells, an established line of hamster fibroblasts, were grown to confluence, labelled with [32 P]P_i (The Radiochemical Centre, Amersham, Bucks., U.K.), their ribosomes isolated, dissociated into subunits, and the protein extracted from these, all as previously described (Leader *et al.*, 1976). In certain experiments the cells were incubated with 2 mM-cyclic AMP (obtained from P-L Biochemicals, Milwaukee, WI, U.S.A.) and 2.5 mM-theophylline [Sigma (London) Chemical Co., Poole, Dorset, U.K.], or with 50 mM-2-deoxyglucose (British Drug Houses, Poole, Dorset, U.K.) after starvation of glucose for 18 h.

Male Wistar rats (300 g) were subjected to surgical removal of 67% of their liver, killed 3 h later and ribosomes isolated from the remaining portion of the liver (Leader, 1975).

Ribosomal proteins were analysed by using the general method of two-dimensional polyacrylamide-gel electrophoresis of Kaltschmidt & Wittmann (1970). This involves separation in the first dimension mainly on the basis of charge, and separation in the second dimension mainly on the basis of size. The detailed modifications of Lastick & McConkey (1976) were adopted for this work and electrophoresis was performed in a miniaturized apparatus (Leader, 1975). The proteins were generally dissolved in a sample buffer containing 8M-urea, 10mM-NH₄HCO₃ and 1mM- β -mercaptoethanol (Howard *et al.*, 1975), except where stated to the contrary.

In experiments where the 40S ribosomal subunits were treated with alkaline phosphatase before extraction of protein, this was as previously described (Leader *et al.*, 1978).

Results and discussion

Our previous studies on the phosphorylation of ribosomal protein S3 (Leader & Coia, 1978a) were performed before it became clear that the protein spot designated as S3 (Sherton & Wool, 1972) in fact contains two proteins, S3 and S3a (Collatz *et al.*, 1977), these not normally being resolved on the two-dimensional gel-electrophoretic system of Kaltschmidt & Wittmann (1970). We have argued from molecular-weight considerations that the phosphorylated protein was, in fact, S3 (Leader, 1980), and this has been confirmed by analysis of the [³²P]P_i-labelled protein on a gel-electrophoretic system similar to System I of Madjar *et al.* (1979),

which does resolve proteins S3 and S3a (D. P. Leader, A. A. Coia & M. J. McGarvey, unpublished work). However, as proteins S3 and S3a were not resolved in the work of Anderson *et al.* (1975), and are not resolved on the similar electrophoretic gels presented here, the spot under consideration here is referred to by its original designation, protein S3, throughout.

We had performed a considerable number of two-dimensional gel-electrophoretic analyses on eukaryotic ribosomal proteins without observing modified forms of ribosomal protein S3 until we came to examine protein from BHK cells that had been subjected to certain adverse conditions. Thus, in the course of studies in which cyclic AMP and theophylline were added to the cells in order to stimulate the phosphorylation of ribosomal protein S6 (Leader & Coia, 1978b), we sometimes observed the response illustrated in Fig. 1. Not only were the characteristic multiphosphorylated derivatives of protein S6 (Gressner & Wool, 1976) not manifest on the two-dimensional gel electrophoretogram, but a new stained spot was visible at the cathodic side of ribosomal protein S3 (there was also an additional spot in a similar position with respect to protein S4). This type of electrophoretic pattern has been observed in three out of six experiments under these conditions, in ribosomal proteins from BHK cells treated with 2-deoxyglucose (Fig. 2), and occasionally in ribosomal proteins from the livers of mice carrying the Krebs II ascites tumour (results not shown). Careful comparison of these results with those of Anderson *et al.* (1975) reveals a strong similarity to the patterns that the latter authors had

Fig. 1. Effect of cyclic AMP on the ribosomal proteins of BHK cells

Protein (200 μ g) from the 40S subunit of the ribosomes of BHK cells was subjected to two-dimensional gel electrophoresis and the proteins were stained with Coomassie Brilliant Blue. The illustration is of protein from (a) control cells and (b) cells incubated for 3 h with 2mM-cyclic AMP and 2.5mM-theophylline. Similar results were also obtained after a 1 h treatment with cyclic AMP. In this and subsequent Figures the positions of the various derivatives of protein S3 are indicated by arrows, the unmodified species bearing the actual designation S3.

Fig. 2. Effect of 2-deoxyglucose on the ribosomal proteins of BHK cells

BHK cells were deprived of glucose for 16 h and then incubated for 3 h with 50 mM-2-deoxyglucose and 10 mCi of [32 P]P_i. Protein (500 μ g) from the 40S ribosomal subunits of these cells was subjected to two-dimensional gel electrophoresis, stained, dried and a radioautograph prepared (Rankine *et al.*, 1977). The illustration shows (a) the pattern of proteins stained with Coomassie Brilliant Blue, and (b) the section of the radioautograph corresponding to the upper part of (a). The positions of the arrows are identical in frames (a) and (b). Some contaminating histones are apparent in (a) and presumably reflect nuclear damage resulting from the treatment with 2-deoxyglucose.

obtained in rat liver at 2 and 4 h after partial hepatectomy. In the electrophoretogram of ribosomal protein from regenerating liver there is not only a spot in a similar position in relation to protein S3, but a cathodically migrating derivative of protein S4 (though unremarked by Anderson *et al.*, 1975) is also clearly present. [Some other differences are apparent in Fig. 1. These are less marked than the differences in proteins S3 and S4 and do not always accompany the latter. For these reasons, and because Anderson *et al.* (1975) presented only

portions of their gels with many proteins excluded, these other differences will not be considered further here.]

In our studies in Krebs II ascites cells the phosphorylated form of protein S3, which occurs when the cells are starved of glucose, appeared to migrate more anodically than does the unmodified form in Fig. 1 (Leader & Coia, 1978a), suggesting that the cathodically migrating modified form was unlikely to be a dephosphorylated derivative of protein S3. However, we could not be certain of this, because of the differences in the electrophoretic conditions [our earlier work (Leader & Coia, 1978a) employed a pH of 6.6, rather than 8.7, in the first dimension] and cells used. In fact the BHK cells from which the ribosomal proteins of Fig. 1 were derived had been incubated with [32 P]P_i for 3 h before being harvested, but no radioactivity was found associated with protein S3 (results not shown). This is consistent with the lack of radioactivity in protein S3 of rat liver after a 20 min pulse of [32 P]P_i (Gressner & Wool, 1974). In an attempt to label protein S3 in BHK cells, we starved the latter of glucose and incubated them with [32 P]P_i. However, under these conditions, and also when the cells were further treated with 2-deoxyglucose (Fig. 2), only very slight labelling of this protein could be obtained (in contrast with the labelling of protein S6, where approx. 1.4 mol of phosphate was incorporated/mol of 40S subunit). Nevertheless, this labelling, which is just visible on the radioautograph of the (deliberately overloaded) gel shown in Fig. 2, was clearly associated with the anodic side of the unmodified ribosomal protein S3 (where there was only a small proportion of the total stain associated with this protein) rather than with the protein itself. Stoichiometrically insignificant labelling of this type would also appear to account for some occasional reports of the phosphorylation of protein S3 in normal cells (Traugh & Porter, 1976; Marvaldi & Lucas-Lenard, 1977; Roberts & Ashby, 1978).

It is still possible to argue that the anodically migrating derivative of protein S3 is a di-phosphorylated species, and that the normal form of protein S3 represents a mono-phosphorylated derivative that it is difficult to label with [32 P]P_i because of a slow rate of phosphate turnover. This seems unlikely on two counts. Firstly, we have labelled the ribosomal proteins of BHK cells for up to 18 h and have still not found incorporation of [32 P]P_i into protein S3 (A. D. Rankine & D. P. Leader, unpublished work). Furthermore, treatment of 40S ribosomal subunits with alkaline phosphatase did not generate any material to the cathodic side of protein S3 (results not shown).

Although changes in other possible physiological chemical modifications, such as acetylation (Liew & Gornall, 1973), were not excluded, it seemed that the

cathodically migrating derivative of protein S3 might be of a type that could be generated as an artifact, as similar species have occasionally been observed in the gel-electrophoretic patterns of ribosomal proteins from normal cells (Welfle *et al.*, 1972; Schiffman & Horak, 1978). A species, S3b, thought to be a proteolytic fragment of protein S3a has been described (Collatz *et al.*, 1977). Although this migrates on the cathodic side of protein S3 during the first dimension of electrophoresis, it migrates further than protein S3 in the second dimension, and thus does not appear to correspond to the species shown in Figs. 1 and 2. Moreover, inclusion of

1 mM-phenylmethanesulphonyl fluoride in the solutions used to isolate ribosomes from BHK cells did not prevent the appearance of the species found here. It was known, however, that ribosomal protein S3 is the protein of the 40S subunit that is most reactive towards *N*-ethylmaleimide (Ücer *et al.*, 1975), and so the possibility that the cathodically migrating derivative of protein S3 might have been generated by the oxidation of cysteine thiol groups was considered.

There was reason for entertaining this idea, because the buffer used to dissolve the ribosomal proteins was prepared at a concentration of β -mercaptoethanol of only 1 mM, and this could have been further decreased by evaporation. A comparison was therefore made between a sample of protein dissolved in this buffer, and a second portion of the same sample dissolved in a buffer containing 0.7M- β -mercaptoethanol (Lastick & McConkey, 1976) and incubated at 60°C for 10 min before electrophoresis. Fig. 3 shows that the cathodically migrating derivatives of both proteins S3 and S4 were almost completely eliminated by the reductive procedure. These latter must therefore be oxidized

Fig. 3. Effect of pre-reduction on the ribosomal proteins of BHK cells treated with cyclic AMP

Protein (200 μ g) from the 40S subunits of the ribosomes of BHK cells incubated as in Fig. 1(b) was subjected to two-dimensional gel electrophoresis after either (a) dissolution in a buffer (Howard *et al.*, 1975) containing 1 mM- β -mercaptoethanol (or less), or (b) dissolution in a buffer (Lastick & McConkey, 1976) containing 0.7M- β -mercaptoethanol, and incubation at 60°C for 10 min. The illustrations are of proteins stained with Coomassie Brilliant Blue.

Fig. 4. Effect of partial hepatectomy on the ribosomal proteins of rat liver

Protein (200 μ g) from the 40S subunits of the ribosomes of the remaining portion of rat liver, 3 h after partial hepatectomy, was subjected to two-dimensional electrophoresis and the proteins were stained with Coomassie Brilliant Blue. The phosphorylated derivative(s) of protein S6 is indicated by an unlabelled arrow. Similar results were also obtained in a separate experiment.

derivatives of the parent proteins. We have presented evidence elsewhere (Leader & Mosson, 1980) that cysteine residues of certain ribosomal proteins, including protein S3, are readily oxidized with the formation of intramolecular disulphide bonds, and that this can result in anomalously high mobilities during electrophoretic separation where there is appreciable 'sieving' of molecules. An analogous explanation may also account for the higher mobilities of proteins S3 and S4 in the first dimension (despite the predominantly charge-based separation) because of their relatively large sizes. (The strongly reducing conditions in the interdimensional treatment would prevent such effects being seen in the 'sieving' second dimension.) Alternatively the reduced (but not the oxidized) forms of proteins S3 and S4 may have ionized cysteine residues at the pH, 8.7, of the first dimension, and hence migrate less rapidly towards the cathode.

In view of these results it was decided to re-examine the 40S ribosomal proteins from regenerating rat liver, taking careful steps to ensure the reduction of the proteins before electrophoresis. Under these conditions there was no cathodically migrating derivative of protein S3, even though the anodically migrating phosphorylated derivatives of protein S6 that Anderson *et al.* (1975) had observed at a similar stage of liver regeneration were clearly present (Fig. 4). It cannot, of course, be concluded that the derivative of protein S3 observed by Anderson *et al.* (1975) arose from oxidation of thiol groups either physiologically or as an artifact, but our failure to confirm this result does suggest that it requires reassessment.

The data presented here show that there is now no reason to suppose that ribosomal protein S3 is phosphorylated in normal tissues. However, some comment is required on the phosphorylation of this protein under abnormal conditions. The failure here to obtain more than trace phosphorylation of protein S3 in BHK cells starved of glucose (Fig. 2) calls into question the generality of our previous conclusion, from work in Krebs II ascites cells, that deprivation of glucose is the stimulus for phosphorylation of protein S3 (Leader & Coia, 1978a). Although further work is clearly required, it is possible that the extreme glycolytic metabolism of the ascites cells, in contrast with the more normal oxidative metabolism of BHK cells, could be relevant to the difference in the responses to glucose deprivation of the two cell types.

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