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A 17 kDa-polypeptide Associated with Polyamine-promoted Growth of Cultured Carrot Cells

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Cell dividing activity of cultured carrot was appreciably depressed by methylglyoxal bis-(guanylhydrazone) (MGBG), an inhibitor of polyamine biosynthesis, and this inhibition was reversed by the addition of spermine or spermidine. In the polyamine-treated cells, a 17 kDa-polypeptide was found to be specifically synthesized in the early stage of the recovery of cell growth. This peptide was also found in exponentially growing carrot cells while undetectable in the cells of the stationary phase. These results suggested the possibility that the 17 kDa-polypeptide is involved in certain cellular event(s) in polyamine-promoted growth of cultured carrot cells.

Introduction

It has been well known that polyamines such as putrescine, spermine and spermidine, play important roles in various cellular events, including DNA replication, protein synthesis and morphogenesis, in a wide range of eukaryotes[1,2]. However, little is known about the biochemical events which take place after the incease in cytoplasmic concentration of these polyamines. In higher plant cells, occurrence of polyamines was unequivocally demonstrated, as well as in animals and microorganisms, and their biosynthetic pathways were also well established together with the enzymes regulating metabolism[3-5]. Recently, we have demonstrated[6] that methylglyoxal bis-(guanylhydrazone) (MGBG), an inhibitor of spermine- and spermidine-biosynthesis, inhibited the cell proliferation of cultured carrot (*Daucus carota*) cells, and this MGBG-induced inhibition of cell growth was almost completely reversed by the addition of 0.05 mM of spermine or spermidine to the culture. These results imply that the inhibitory effect of MGBG resulted from the deficiency of necessary polyamines in carrot cells, and spermine and/or spermidine but not putrescine are the essential polyamines for the growth of the cells. It has also been confirmed[6] that the rate of cell proliferation and the intracellular concentration of these polyamines, especially that of spermidine, in cultured carrot are closely correlated with each other. By contrast, Maki *et al.* studied the changes in polyamine levels and enzyme activities involved in their biosynthesis, and concluded that putrescine plays a more important role than other polyamines in cell proliferation of *Catharanthus roseus*[7]. These kinds of contradictory observations on the functions of individual polyamines are sometimes observed in several biological events in higher plant cells[3,5]. These findings suggested the possibility that a certain mediator or mediators, including proteins, are synthesized as a common substance in a wide range of plant species upon the increase in intracellular polyamine level, and this leads polyamines to signal appropriate cellular responses. In the present experiment, we attempted to identify polyamine-specific protein(s) which might be synthesized in the early stage

of the recovery of cell growth evoked by the addition of polyamines to MGBG-treated carrot cell culture.

Materials and Methods

1. *Label-chase of TCA insoluble proteins in cultured carrot cells*

Cultured carrot cells were grown in Murashige and Skoog's synthetic medium[8] in the presence of 2, 4-dichlorophenoxyacetic acid as described previously[9]. Forty m*l* of exponentially growing cell culture (4-day-old) were incubated with 0.5 mM of MGBG for 48 h at 26°C as described previously[6]. In the last 3 h of this MGBG-treatment, cells were radiolabeled with 1.11 MBq of [^{14}C]leucine (New England Nuclear, specific activity 11.5 GBq/mmol). After labeling, cells were washed with 50 m*l* of fresh medium (4 times), and the culture volume was adjusted to 40 m*l*. This labeled culture was divided into 3 portions, and to each culture was added filter-sterilized 0.5 mM of MGBG, MGBG plus 0.05 mM of spermine or MGBG plus 0.05 mM of spermidine, respectively. These cultures were further incubated, and, at regular intervals, 1 m*l*-aliquots were removed. The cells and medium were separated by centrifugation (750×g, 5 min.), and after repeated washing, the volume of the cell suspension was readjusted to 1 m*l* with water. Then, the cells were killed by boiling for 2 min., and were homogenized by sonic oscillation. A portion of this cell homogenate was removed to determine the radioactivity, and the value was referred to as the total incorporation of [^{14}C]leucine into the cells. Cell debris was removed by centrifugation (750×g, 5 min.), and to the resultant supernatants was added 1 m*l* of 10% (w/v) TCA. Precipitated proteins were successively washed with the same volume of TCA (3 times) and ethanol by centrifugation, and their radioactivities were determined.

2. *Analyses of radiolabeled proteins*

Radiolabeled proteins (2.5×10^4 dpm each) were separated by SDS-PAGE (10% gel) according to the method of Laemmli[10], and after electrophoresis, the gel was stained with Coomassie Brilliant Blue. Then, the gel was immersed in Enlightening (New England Nuclear) for 20 min., dried, and exposed to a X-ray film (Kodak, X-OMAT AR) for 7 days at −80°C for fluorographic analyses.

Results and Discussion

In preliminary experiments, changes in the rate of bulk protein synthesis in polyamine-dificient and polyamine-rich carrot cells were determined with time (**Fig. 1**). In order to prepare spermine- and spermidine-deficient carrot cells, exponentially growing cell culture (4-day-old) were incubated in the presence of MGBG, and the proteins in the cells were labeled with [^{14}C]leucine. These cells were further incubated with MGBG, MGBG plus spermine or MGBG plus spermidine, and radioactivities associated with TCA insoluble fractions of the cells were determined. Protein synthesis in carrot cells which had not been previously treated with MGBG initiated without a notable lag, and reached a maximal level after 3 h of chase. By contrast, the protein synthetic rate in the cells cultured with MGBG were appreciably depressed, and about 3 h of lag phase was observed. Maximal synthesis in these cells was achieved after 7 h of the chase. When spermine or spermidine was added to MGBG-treated cell culture immediately before chase, two peaks of protein synthesis (2 and 5 h after the addition of polyamines) in the cells were observed in both treatments. In repeated experiments, these two peaks were reproducibly observed in polyamine-treated carrot cells although the timings were varied to some extent.

In the next experiments, we compared the proteins which were obtained in the peak stage of biosynthesis after the addition of the inhibitor alone or together with polyamines. Radiolabeled proteins (2.5×10^4 dpm each) were separated by SDS-PAGE, and the gel was subjected to protein

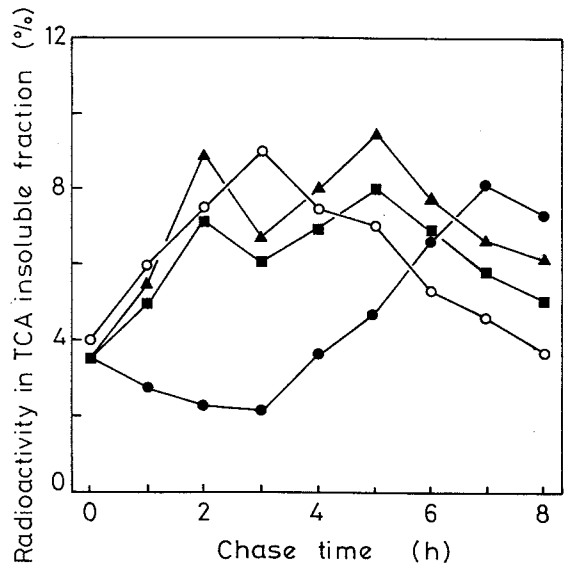

Fig. 1 Change in protein synthetic activities in cultured carrot cells treated with MGBG or polyamines.
Four-day-old carrot cell culture was incubated with MGBG for 48 h, and the cells were radiolabeled with [^{14}C]leucine. After the labeling, cells were washed, and MGBG(●), MGBG plus spermine(■), or MGBG plus spermidine(▲) was added to the culture. At regular intervals, radioactivities associated with TCA-insoluble fraction of the cell homogenates were determined. The results are expressed as percentage to total incorporation of [^{14}C]leucine into respective cell homogenates. As control(○), carrot culture grown without the addition of MGBG or polyamines was label-chased, similarly.

staining or fluorography. As shown in **Fig. 2-a**, the profiles of electrophoresis of the radiolabeled proteins in MGBG-, MGBG plus spermine- or MGBG plus spermidine-treated carrot cells were very similar. However, in the polyamine-treated cells, an appreciable signal was observed at the position corresponding to 17 kDa which was not found in MGBG-treated carrot cells. This 17 kDa-peptide showed the strong signals in the earlier peaks of protein synthesis (about 2 h after the chase) both in spermine- and spemidine-treated cells (lane 2, 4) while, in the later peaks (lane 3, 5), intensities of the band decreased significantly (53% of the earlier peak in spermine- and 45% in spermidine-treated cells as determined by densitometer). Each lane of the gel in **Fig. 2** received the same amount of radioactivity and the protein contents of these samples, as determined by the method of Bradford[10], were also shown to be comparable (33-41 μg). When the gel was stained with Coomassie Brilliant Blue, only a faint band of the 17 kDa-peptide was observed in MGBG-treated cells (**Fig. 2-b**). By contrast, in polyamine-treated cells, the protein band was clearly detected in either an earlier or later peak of protein synthesis, and the intensities of the band were almost comparable in both peaks. Results obtained here indicated that biosynthesis of the 17 kDa-peptide was markedly inhibited in MGBG-treated carrot cells although the synthesis of general proteins in the cells appeared not to be affected by the treatment. In addition, it was also shown that synthesis of the 17 kDa-peptide was specifically observed in the early stage of the recovery of cell dividing activity of carrot by the addition of polyamines. These results led us to hypothesize that the 17 kDa-peptide relates to or is involved in early events of polyamine-promoted cell growth of cultured carrot.

If this 17 kDa-peptide correlates with cell growth of cultured carrot, this peptide should be

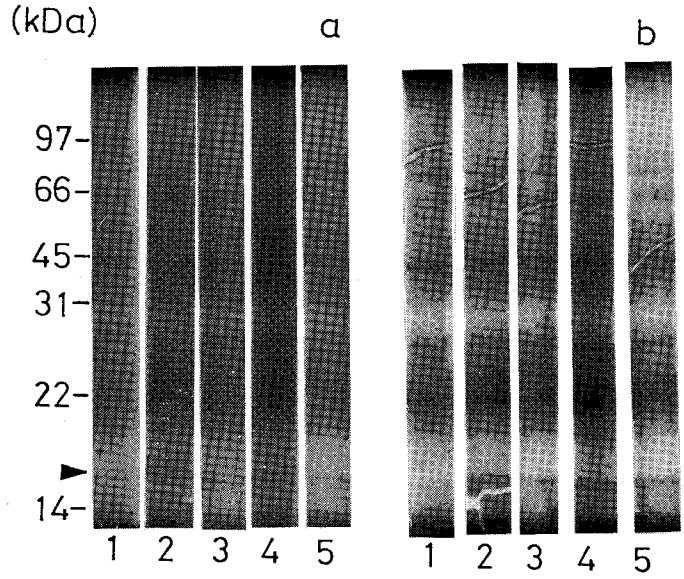

Fig. 2 SDS-PAGE analysis of TCA-insoluble proteins in MGBG- or MGBG plus polyamine-treated carrot cells.
Radiolabeled proteins (2.5×10^4 dpm) obtained at the peak stages of protein synthesis in MGBG (lane 1)-treated, and earlier or later peaks in spermine (lane 2, 3)- or spermidine (lane 4, 5)-treated carrot cell culture were separated by SDS-PAGE, and the gel was subjected to fluorography (a) or protein staining with Coomassie Brilliant Blue (b). Positions of molecular weight markers are given as bars, and the 17 kDa-polypeptide is shown by arrowhead.

appreciably synthesized and exist at a significant concentration in actively growing carrot cells even in the absence of exogenously supplied polyamines. Therefore, changes in synthetic activity and cellular level of the 17 kDa-peptide in various growth stages of cultured carrot cells were examined (**Fig. 3**). Cultured carrot cells in exponential (4-day-old), late logarithmic (8-day-old) and stationary (12-day-old) phases were radiolabeled, and samples obtained from the chase time at which protein synthetic activity was maximal were analyzed as is in **Fig. 2**. When proteins corresponding to 6×10^6 cells (counted under a microscope using a counting chamber) were applied to SDS-PAGE (**Fig. 3-a**, lane 1-3), a weak signal of the 17 kDa-peptide was detected in the exponentially growing carrot cells. By contrast, its synthesis was almost undetectable in late logarithmic and stationary phases. These samples contained much lower levels of proteins though they were obtained equally from 6×10^6 cells, probably because of the progress of senescence in these culture ages. In lane 4 and 5, labeled proteins with the same radioactivity to the sample of lane 1 (2.3×10^4 dpm) were analyzed by fluorography, but, even in this case, the signal of 17 kDa-peptide was undetectable in these growth phases. After the protein staining, the band of the 17 kDa-peptide was able to detect in the homogenates prepared from exponentially growing cells (**Fig. 3-b**, lane 1). By contrast, the peptide could not be detected in cell homogenates from late logarithmic (lane 2) and stationary (lane 3) phases. Even when the same amount of photeins (37 μg each) of different growth stages were applied (lane 1, 4, 5), the 17 kDa-peptide, essentially could not be observed in late logarithmic and stationary phases, as was the protein synthetic activity. These results showed that the 17 kDa-polypeptide is synthesized in carrot cells in a phase specific manner, and the peptide occurs only in the actively growing cells at a significant level.

Results obtained in the present experiments indicated that; 1) a 17 kDa-polypeptide was synthe-

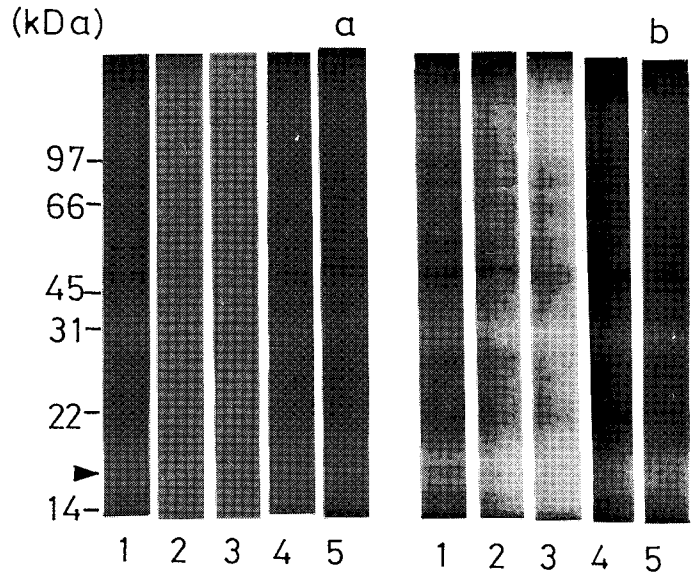

Fig. 3 SDS-PAGE analysis of TCA-insoluble proteins in cultured carrot cells at various growth stages.
Cultured carrot cells in exponential (lane 1), late logarithmic (lane 2, 4) and stationary (lane 3, 5) phases were radiolabeled and chased with time. Samples obtained from the chase time at which protein synthetic activity was maximal were analyzed. (a) Radiolabeled proteins corresponding to 6×10^6 cells (lane 1-3) or of same radioactivity (2.3×10^4 dpm, lane 1, 4, 5) were applied to SDS-PAGE followed by fluorography. Difference of the incorporation efficiency of [^{14}C]leucine in different growth stages was corrected by measuring the radioactivities in the total cell homogenate of each sample. (b) Proteins corresponded to 6×10^6 cells (lane 1-3) or the same amount of proteins (37 μg, lane 1, 4, 5) were separated by SDS-PAGE, and were stained with Coomassie Brilliant Blue.

sized specifically in the early stage of the recovery of cell proliferation caused by the addition of spermidine or spermine to MGBG-treated carrot cells; 2) the 17 kDa-peptide was commonly synthesized in both spermine- and spermidine-treated carrot cells; 3) synthesis of the 17 kDa-peptide takes place not only in the recovery stage of MGBG-treated carrot cells with polyamines but also in normally growing cells in the exponential phase. These observations suggested that the 17 kDa-polypeptide possibly plays a role in cell growth of cultured carrot in which spermine or spermidine is an essential factor.

From the present experiments, the possibility cannot be ruled out that the 17 kDa-peptide is a protein involved in polyamine metabolism, for example, a degradation enzyme of these amines. However, the 17 kDa-peptide was synthesized as a common response upon the addition of different species of polyamines, spermine and spermidine, and therefore, it is unlikely that the peptide is merely a metabolic enzyme of polyamines. Intensities of earlier peaks of protein synthesis in polyamine-stimulated carrot cells (**Fig. 1**) seems to be significantly high compared with the signals of 17 kDa-peptide in fluorographic analysis (**Fig. 2-a**) suggesting that the early enhancement of protein synthesis in polyamine-treated carrot cells is not restricted to the 17 kDa-peptide. Therefore, it is reasonable to expect that several polypeptides other than the 17 kDa-peptide are also synthesized and maintained in carrot cells as cell growth-related proteins of which metabolism is controlled by polyamines. Further characterization and survey of the distribution of the 17 kDa-peptide in other plant sources are in progress in our laboratory.

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《和文要約》

ポリアミンによって誘導されるニンジン培養細胞の分裂とそれに関わる 17 kDa のポリペプチド

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ニンジン培養細胞の分裂能はポリアミンの生合成阻害剤 methylglyoxal bis-(guanylhydrazone) (MGBG) を添加することによって著しく阻害されるが，MGBG 存在下であってもスペルミンやスペルミジンを加えると細胞分裂が開始される．このポリアミン添加による細胞分裂の回復の初期の段階で，17 kDa のペプチドが特異的に合成されることが観察された．対数増殖期にあるニンジン細胞にもこのペプチドが存在することが確認できたが，定常期の細胞には見いだされなかった．これらの結果から，ポリアミンによって誘導される細胞分裂のなんらかの過程にこの 17 kDa のペプチドが関わっている可能性が示唆された．