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Identification of phosphoproteins correlated with proliferation and cell cycle arrest in *Saccharomyces cerevisiae*: Positive and negative regulation by cAMP-dependent protein kinase

(yeast/phosphorylation/phosphoamino acid/nutritional sensing)

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ABSTRACT **Recent genetic and biochemical studies of two mutants of the cAMP pathway in yeast, cyr1 and bcy1, have demonstrated that cAMP-dependent protein phosphorylation plays a major regulatory role in the control of proliferation and differentiation. As a first step in examining this regulatory system in more detail and in identifying the protein substrates of cAMP-dependent protein kinase, we have analyzed phosphoprotein patterns in the mutants cyr1-2(ts) and bcy1 by two-dimensional polyacrylamide gel electrophoresis. Our analysis has revealed several proteins whose phosphorylation is controlled positively or negatively by the cAMP pathway in yeast. The presence of some of these phosphoproteins was directly associated with proliferation (positive regulation), while that of others was correlated with cell cycle arrest (negative regulation). The phosphoprotein patterns of cyr1-2(ts) temperature-arrested cells, and nitrogen (NH_4^+)-starved cells, were strikingly similar, suggesting that response to NH_4^+ is mediated in part by adenylate cyclase. Phosphoproteins whose presence correlated with cell cycle arrest were found to be phosphorylated on serine and threonine residues, while the major phosphoproteins present predominantly in proliferating cells were phosphorylated only on serine residues. None of the >20 phosphoproteins we examined contained phosphotyrosine under either growth condition.**

In *Saccharomyces cerevisiae*, the cAMP pathway appears to play an essential role in controlling the transition between proliferation (vegetative growth) and differentiation (meiosis and ascospore formation) (1, 2). Since this transition is normally controlled by nutritional signals (3, 4), the cAMP pathway is a critical component in mediating the developmental response to nutritional information. These conclusions are based in large part on the analysis of two mutants, cyr1 and bcy1. The mutant *cyr1-2(ts)*, expressing a thermolabile adenylate cyclase, undergoes G_1 arrest at the restrictive temperature similar to other *cdc* start mutants (1). Mutations at *CYR1* are suppressible by mutations at another locus, *BCY1*. The lesion in the mutant, bcy1, results in a deficiency of the regulatory subunit of cAMP-dependent protein kinase, rendering cAMP-dependent protein kinase activity constitutive and independent of cAMP (5). The major conclusions drawn from the analysis of *cyr1* and *bcy1* are that (*i*) cAMP-dependent protein phosphorylation is required for cell cycle initiation and vegetative growth, and (*ii*) cAMP-dependent protein kinase must be inactivated before cell cycle arrest or sporulation can take place; that is, cAMP-dependent protein kinase exerts a positive control over proliferation, and a negative control over differentiation (regulated cell cycle arrest and sporulation).

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Our objective in this study was to begin an analysis of the components of this regulatory pathway by examining the phosphoprotein profile in the mutants cyr1-2(ts) and bcy1 with the resolution that two-dimensional gel electrophoresis provides. Our approach has been to study mutant and wild-type cells under proliferation conditions (or in which cAMP-dependent protein kinase is active), and under conditions that lead to cell cycle arrest. This analysis has led to the identification of different classes of proteins whose phosphorylation appears to be regulated positively and negatively by cAMP-dependent protein kinase.

MATERIALS AND METHODS

Yeast Strains. Diploid strains X282 (**a**/α *his7/his7 +/leu1 bcy1/bcy1*), X286 [**a**/α *cyr1-2(ts)/cyr1-2(ts)*)], and X287 (**a**/α *leu1/+ cyr1-2(ts)/+*) were used in this study. All three were kindly provided by K. Matsumoto.

Media and Labeling Conditions. Radioactive $^{32}PO_4$ labeling of cells was carried out in the low phosphate medium, CM or NCM, with 1% glucose (6). Noncycling medium (NCM) is CM without NH_4Cl. Cells (10 ml) were labeled with [^{32}P]orthophosphate (ICN; carrier free) (200 μCi/ml; 1 Ci = 37 GBq) at 28°C, or at 36°C, the restrictive temperature for *cyr1-2(ts)* (strain X286).

Protein Extraction and Two-Dimensional Gel Electrophoresis. The cells were broken with glass beads, and the protein was extracted and lyophilized as described by Wright *et al.* (7). The protein pellet was resuspended in 50 μl of a solution containing 9.5 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, 0.5% ampholytes (pH 3–10), and 1% ampholytes (pH 5–7) (LKB) and subjected to two-dimensional gel electrophoresis as described by O'Farrell (8). Focusing in the first dimension was carried out by running at 400 V for 16 hr, followed by 800 V for 1.5 hr. Standard 10% acrylamide gels were run in the $NaDodSO_4$ dimension. After electrophoresis, the gels were fixed, stained, and dried as described by O'Farrell (8).

Phosphoamino Acid Analysis. Individual spots were localized on the gel by autoradiography after exposures of 2–10 hr, cut out and combined from three or four gels, extracted, acid-hydrolyzed, and the resultant hydrolysate was analyzed by two-dimensional thin-layer electrophoresis as described by Cooper *et al.* (9).

RESULTS AND DISCUSSION

Rationale. We reasoned that the phosphoprotein pattern of cyr1-2(ts) cells labeled at the restrictive temperature should lack the subset of proteins normally phosphorylated by

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cAMP-dependent protein kinase, but it should still contain proteins that are phosphorylated by protein kinases other than cAMP-dependent protein kinase (unless such protein kinases are activated by cAMP-dependent protein kinase). Conversely, the phosphoprotein pattern of cells in which adenylate cyclase is active should represent a complete set of proteins associated with proliferating cells, including the substrates of cAMP-dependent protein kinase. Given the putative role of NH_4^+ as a signal molecule for proliferation and differentiation (3, 4), we would also expect that the phosphoprotein pattern of temperature-arrested *cyr1-2(ts)* cells, in the presence of NH_4^+, should be similar to that of NH_4^+-starved and arrested wild-type cells. Moreover, if cAMP-dependent protein kinase were the primary mediator of the cAMP signal, then *bcy1* cells, which do not respond to NH_4^+ deprivation, should not show the NH_4^+ starvation response with respect to their phosphoprotein patterns.

In our first study, we examined and compared the two-dimensional polyacrylamide gel electrophoretic phosphoprotein patterns of a *cyr1-2(ts)* homozygous diploid (strain X286; Table 1) with a closely related diploid heterozygous for *cyr1-2(ts)*, referred to in the following discussion as *CYR1* (strain X287). Cells were labeled with [^{32}P]orthophosphate at 28°C or at 36°C, a temperature restrictive for growth of

Table 1. Relative level and phosphoamino acid content of phosphoproteins

	cyr1-2(ts) arrested (36°C)	*CYR1* (NH_4^+-starved)	*CYR1* proliferation (36°C)	*bcy1* proliferation and NH_4^+-starved	Phosphoamino acid
Group 1: Phosphoproteins in low abundance in *cyr1-2(ts)* temperature-arrested and wild-type NH_4^+-starved cells					
a	−	−	+++++	+++++	S
b	−	−	+++++	+++++	S
h	−	−	+++	++	S
f	−	+	+++++	++	S: proliferation S,T, Ψ: NH_4^+-starved
J region	+/−	+/−	+++++	+++++	S, trace of T: proliferation S,T: NH_4^+-starved
Group 2: Phosphoproteins in low abundance in proliferating cells					
c	+++++	+++++	−	−	S,T, Ψ
d	+++++	+++++	−	−	S,T, Ψ
o	+++++	+++++	−	−	S,T
p	+++++	+++++	−	−	S,T, Ψ
s	+++++	+++	−	−	S,T, Ψ
t	+++++	+++++	+	−	S,T, Ψ
v	+++++	+++++	+	−	S: proliferation S,T: NH_4^+-starved
w	+++++	+++++	+	−	S,T
z	+++++	+++++	+	−	S,T
Group 3: Phosphoproteins in low abundance in *bcy1* cells					
q	+++++	+++++	+++++	−	S,T
u	++	++++	+++	−	S,T
m	+++++	+++	++++	+	S,T
n	++++	++++	+++	+	S,T
y	+++	+++	+++	+	S,T, Ψ
r	+++++	+++++	++++	+	S: proliferation S,T: NH_4^+-starved
K region	+++++	++	++	+/−	S,T, Ψ: NH_4^+-starved
Group 4: Phosphoproteins in low abundance in NH_4^+-starved wild-type cells					
e	+++	+	+++++	++++	S: proliferation S,T, Ψ: NH_4^+-starved
Reference phosphoproteins detected in all conditions and strains					
g	+++	+++	+++++	+++++	S: proliferation S,T: NH_4^+-starved
x	+++++	+++++	++++	+++	S,T: NH_4^+-starved

The relative levels of the different spots were estimated visually by scoring different exposures (1–48 hr). Comparisons between different experiments and strains were done by normalizing to the reference proteins g and x. Unless otherwise indicated, the phosphoamino acid content is given for the condition in which the given phosphoprotein is most prominent. For a few, the phosphoamino acid content was examined under proliferation and NH_4^+-starvation conditions. S and T represent phosphoserine and phosphothreonine, respectively. In all cases examined (except for the K region proteins), S was always greater than T. Ψ is the compound that migrates very close to the phosphotyrosine marker.

cyr1-2(ts) but not for *CYR1*. Several labeling intervals and durations (0–15, 0–30, 15–30, 0–60, 30–60, 60–90, 60–120, 120–180, 120–240, 240–300, 240–360, 300–360, and 360–480 min after temperature upshift) were used. We observed similar qualitative results for each strain, regardless of the labeling interval or duration. By 240–360 min after temperature upshift to 36°C, >90% of *cyr1-2(ts)* cells were arrested as unbudded cells. Their two-dimensional phosphoprotein pattern is shown in Fig. 1*B*. *CYR1* cells, on the other hand, were growing exponentially. The phosphoprotein patterns of *cyr1-2(ts)* cells labeled at 28°C and of *CYR1* cells labeled at either 28°C (not shown) or 36°C (Fig. 1*A*) were the same.

In the second set of experiments, we analyzed the phosphoprotein pattern of *cyr1-2(ts)*, *CYR1*, and *bcy1* (strain X282) and wild-type cells in response to NH_4^+ depletion at both 28°C and 36°C. Representative gels for NH_4^+-starved and -arrested *CYR1* cells (36°C) and NH_4^+-starved *bcy1* cells also at 36°C are shown in Fig. 2. The phosphoprotein pattern for

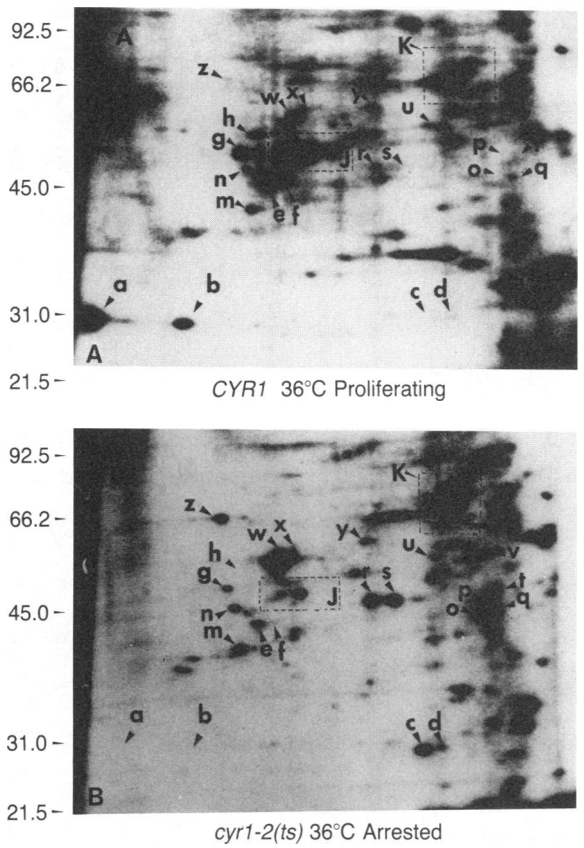

FIG. 1. Two-dimensional gel electrophoretic analysis of phosphoproteins isolated from proliferating *CYR1* cells, and temperature-arrested *cyr1-2(ts)* cells. (*A*) Phosphoprotein pattern representative of proliferating *CYR1* cells. (*B*) Phosphoprotein pattern representative of temperature-arrested *cyr1-2(ts)* cells. *CYR1* (X287) and *cyr1-2(ts)* (X286) cells were grown in the low phosphate medium CM/1% glucose at 28°C to a cell density of 2–3 × 10^6 cells per ml. The *cyr1-2(ts)* cells were then shifted to 36°C and incubated for 6 hr and labeled (cells arrested at 5–6 × 10^6 cells per ml) for 2 hr with [^{32}P]orthophosphate (200 μCi/ml). The *CYR1* cells were similarly treated except that the cell density was adjusted to 2–3 × 10^6 cells per ml at the time of labeling and to 5–6 × 10^6 cells per ml at the time of harvest. The cells were then rapidly harvested by filtration, washed three times with 10 ml of ice-cold distilled water, and then frozen at 70°C. The cells were broken as described in *Materials and Methods*, and proteins were extracted and resolved by two-dimensional gel electrophoresis. Approximately equal amounts of protein were loaded on each gel. Isoelectric focusing in the first dimension is from left (basic, pH ≈7.4) to right (acidic, pH 5.4). Autoradiography was performed with an intensifying screen (*A*, 8-hr exposure; *B*, 10-hr exposure).

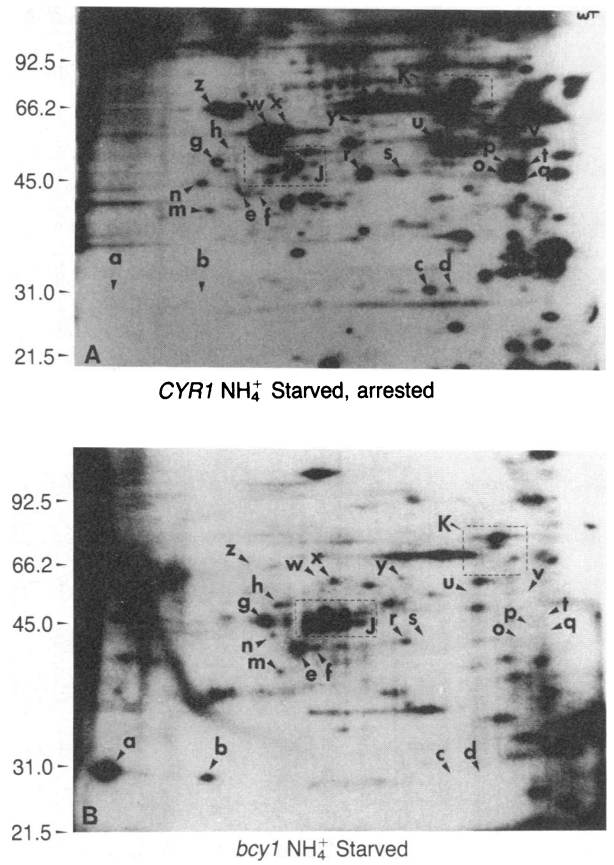

FIG. 2. Two-dimensional gel electrophoretic analysis of phosphoproteins isolated from NH_4^+-starved cells. (*A*) Phosphoprotein pattern representative of *CYR1* NH_4^+-starved cells. (*B*) Phosphoprotein pattern representative of *bcy1* NH_4^+-starved cells. *bcy1* cells do not arrest at a unique stage on the cell cycle after nutritional deprivation. *CYR1* (strain X287) and *bcy1* (strain X282) cells were grown in CM/1% glucose to 2–3 × 10^6 cells per ml, and then shifted to NCM/1% glucose. The cells were incubated for 6 hr in NCM (> 90% of *CYR1* cells were unbudded by 4 hr; ≈50% of the *bcy1* cells were budded), and their densities were adjusted to 5–6 × 10^6 cells per ml. The cells were labeled for 2 hr, harvested, and protein was extracted and resolved by two-dimensional gel electrophoresis as described above. (*A*, 8-hr exposure; *B*, 8-hr exposure).

NH_4^+-starved *CYR1*, *cyr1-2(ts)*, and wild-type cells was the same at both temperatures (data not shown). The results of these experiments are summarized in Table 1.

In both the first and second set of experiments, we tested for the effects of phosphatases by including the phosphatase inhibitors, sodium vanadate and sodium fluoride (final concentration, 10 mM) in the cell breaking buffer. In another series of controls, the cells were boiled for 2 min in the breaking buffer, and then broken at 90°C–100°C. No differences in any of the phosphoprotein patterns were observed, suggesting that protein phosphatase action was minimal under our extraction conditions. Incubation of the cell lysate with proteinase K eliminated all spots in the gels, showing that all the labeled compounds were proteins.

Proliferation and Cell Cycle Arrest Phosphoproteins. Our analysis clearly identifies proteins whose phosphorylation is associated with either the proliferating or the nonproliferating state. It is likely that the phosphorylation of these proteins is regulated by changes in activity of protein kinases and phosphatases rather than in the availability of substrates. However, until the unphosphorylated forms of these phosphoproteins are identified and quantified under various conditions, our interpretation cannot be proved. Based on their phosphorylation phenotype, these proteins were divided into four groups. Those in group 1 (a, b, h, f, and those in

region J) were not detected or were barely detected in nonproliferating cells—i.e., *cyr1-2(ts)* temperature-arrested cells, as well as *CYR1* cells arrested by NH_4^+ starvation. On the other hand, they were prominent in proliferating cells (Fig. 1*A*) and in cells in which cAMP-dependent protein kinase activity is constitutive (and not subject to cAMP regulation)—i.e., *bcy1* cells cultured under nutritionally sufficient conditions (not shown), as well as in *bcy1* NH_4^+-starved cells (Fig. 2*B*). These proteins are either substrates of cAMP-dependent protein kinase or, alternatively, their phosphorylation is positively regulated by cAMP-dependent protein kinase. Their presence may be necessary, but it is not sufficient for cell cycle initiation and vegetative growth.

An inverse correlation was observed for group 2 proteins (c, d, o, p, s, t, v, w, and z). These phosphoproteins were very prominent in nonproliferating cells: *cyr1-2(ts)* at 36°C (Fig. 1*B*), or NH_4^+-starved and arrested *cyr1-2(ts)* (not shown) and *CYR1* cells (Fig. 2*A*). However, they were not detected or were barely detected in proliferating cells (Fig. 1*A*) or in *bcy1* cells (Fig. 2*B*). The phosphorylation of this group of proteins appears to be regulated negatively by cAMP-dependent protein kinase. We cannot determine from these data whether this is accomplished through another protein kinase, itself inactivated by cAMP-dependent protein kinase, or through a phosphatase that is activated by cAMP-dependent protein kinase. The absence of group 2 proteins in NH_4^+-starved *bcy1* cells underscores the necessity of distinguishing between regulated (i.e., inactivation of cAMP-dependent protein kinase and/or ability to respond to NH_4^+ deprivation) and nonregulated (i.e., as seen in *bcy1* cells) growth arrest.

Group 3 (q, u, m, n, y, r, and region K) consists of phosphoproteins that are absent or barely detectable in *bcy1* cells but are otherwise found in both proliferating and nonproliferating cells. It is important to point out that the phosphoprotein pattern in *bcy1* cells is the same whether the cells are in nutrient medium or have been starved for NH_4^+. The existence of this group is intriguing and indicates a difference between cells in which the activity of cAMP-dependent protein kinase is regulated (*CYR1*) and those in which it is not (*bcy1*). Although we are tempted to ascribe their paucity in *bcy1* cells to the fact that the activity of the catalytic subunit of cAMP-dependent protein kinase is constitutive, we cannot exclude the possibility that it is due instead to the low concentration of the regulatory subunit. Possibly, the level of phosphorylation of a large number of proteins by other protein kinases is tightly (and negatively) regulated by cAMP-dependent protein kinase. For the proteins of this group, a constitutive (and perhaps more active) cAMP-dependent protein kinase may result in their reduced phosphorylation. Since *bcy1* cells do not undergo a regulated arrest after nutritional deprivation, perhaps the phosphorylation of this group of proteins is essential for the cell to respond to growth-arrest signals appropriately. The phosphorylation of proteins in region K appears to be negatively controlled by cAMP-dependent protein kinase. However, their phosphorylation can also apparently be controlled independently of cAMP-dependent protein kinase, since in NH_4^+-starved and arrested cells the phosphorylation of proteins in this region is similar to that in proliferating cells.

Group 4 (phosphoprotein e) is of interest since it was preferentially deficient in NH_4^+-starved *cyr1-2(ts)* (not shown) and *CYR1* (Fig. 2*A*) cells, but it was abundant in *bcy1* cells (starved or not), *CYR1* proliferating cells, and in *cyr1-2(ts)* temperature-arrested cells. Apparently either NH_4^+ sufficiency or constitutive cAMP-dependent protein kinase activity leads to the phosphorylation of protein e. Hence, while the appearance of group 2 phosphoproteins when adenylate cyclase is inactive or under conditions of NH_4^+ deprivation indicates that adenylate cyclase is involved in mediating NH_4^+ nutritional information, the low prominence of phosphoprotein e in NH_4^+-depeleted cells argues that not all NH_4^+ nutritional information is mediated by the cAMP pathway.

Phosphoamino Acid Content of Phosphoproteins. Most of the phosphoproteins of interest were analyzed for their phosphoamino acid content after extraction from two-dimensional gels of ^{32}P-labeled proteins from either proliferating or arrested cells, and our results are summarized in Table 1. Four examples of this analysis are shown in Fig. 3. All of the proteins were found to contain phosphoserine. The group 1 phosphoproteins contained only phosphoserine, apart from region J, which had a trace of phosphothreonine. The phosphoamino acid compositions of the group 1 proteins is consistent with their phosphorylation being carried out by cAMP-dependent protein kinase, which modifies proteins mainly on serine residues (10) (e.g., phosphoprotein a in Fig. 3*A*). Group 2 phosphoproteins also contained predominantly phosphoserine, but in every case they also contained detectable phosphothreonine (e.g., phosphoproteins c and d in Fig. 3 *C* and *D*). An increase in phosphothreonine content was also observed in those proteins in groups 1, 3, and 4 that could be detected in arrested cells. This implies either that a protein kinase specific for threonine is more active in arrested cells or that a phosphothreonine-specific phosphatase is inactivated. Group 3 phosphoproteins also contained mostly phosphoserine, except for region K spots, which are unusual in

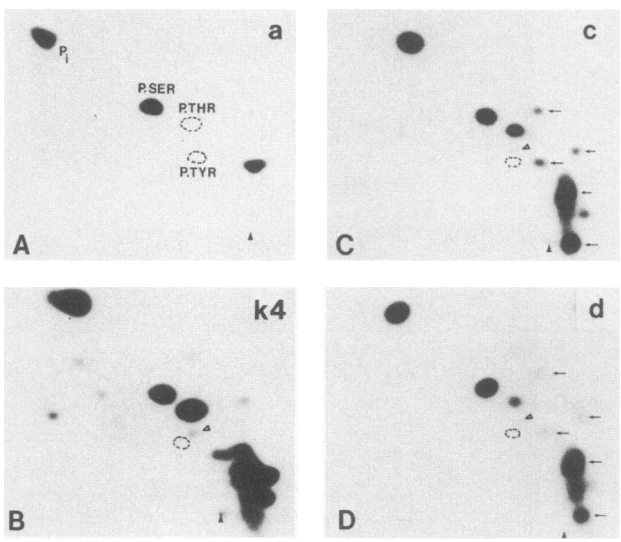

FIG. 3. Phosphoamino acid analysis of representative phosphoproteins prominent in proliferating or nonproliferating *CYR1* cells. Phosphoproteins a and spot K4 from region K (*A* and *B*), and phosphoproteins c and d (*C* and *D*) were cut out from 4 two-dimensional polyacrylamide gels of ^{32}P-labeled proteins from *CYR1* cells labeled as described under the pertinent growth conditions. Phosphoamino acids were generated from extracted proteins by partial acid hydrolysis and were resolved by two-dimensional thin-layer electrophoresis as described (9). Electrophoresis was performed at pH 1.9 in the first dimension (horizontal) (origins indicated by closed arrowheads) toward the anode (on the left) and at pH 3.5 in the second dimension (vertical). The following amounts of Cerenkov radioactivity were analyzed: protein a, 180 cpm; protein K4, 1500 cpm; protein c, 380 cpm; protein d, 320 cpm. The plates were exposed to presensitized Kodak XAR film with an intensifying screen at −70°C for 3 days. The positions of phosphoserine (P.SER), phosphothreonine (P.THR), phosphotyrosine (P.TYR), and orthophosphate (P_i) are given in *A*. Where no radioactivity was detected, the position of the marker phosphoamino acid is indicated by a dashed circle. Protein a, typical of all proteins examined, contained phosphoserine. Proteins c, d, and K4 from NH_4^+-starved cells contained phosphothreonine as shown here. Open arrowheads indicate the position of the Ψ spot, which runs very close to phosphotyrosine. Horizontal arrows show the pattern of phosphopeptides released after partial acid hydrolysis.

that they had relatively high levels of phosphothreonine. For example, protein K4 (excised from the highest molecular weight part of the K region) contains more phosphothreonine than phosphoserine in NH_4^+-starved *CYR1* cells (Fig. 3*B*).

Some information about the possible relatedness of different phosphoproteins was obtained by examining the patterns of partial hydrolysis products, which are often characteristic for a given protein. We focused our attention on proteins that had similar second dimension mobilities or that were clustered. For example, the similarity of the patterns of phosphopeptides of proteins c and d (Fig. 3 *C* and *D*, horizontal arrows) suggests that these two proteins are related. All the J region proteins appeared to be related to each other and possibly to phosphoprotein g. Phosphoproteins r and s were related but distinct from the J proteins. All the region K proteins were related, as were the o, p, q, and t quartet. Other pairs of proteins that appeared to be related are a and b, e and f, and w and x. It seems likely that related proteins represent charge isomers due to the presence of multiple phosphorylation sites. None of the 35 spots (20 or so different phosphoproteins) examined contained detectable amounts of phosphotyrosine, despite recent evidence that phosphotyrosine can be detected in yeast hydrolysates after *in vivo* labeling with [^{32}P]orthophosphate (11), or that activities capable of phosphorylating tyrosine-containing peptides *in vitro* can be detected in yeast extracts (12). Several of the phosphoprotein hydrolysates contained a radioactive compound (Ψ), which migrated very close to the phosphotyrosine marker (Fig. 3 *B–D*, open arrowheads). We have observed a similar compound in hydrolysates of some phosphoproteins from mammalian cells, whereupon further analysis it proved to be derived from phosphoserine (9). We have not examined this compound from a yeast protein, but it is interesting to note that it was much more frequently observed in hydrolysates of phosphoproteins isolated from arrested cells.

A number of other relatively acidic ^{32}P-labeled species were released by acid hydrolysis from several of the yeast phosphoproteins (e.g., the spots migrating below P_i in Fig. 3*B*), which are not typical of hydrolysates of mammalian phosphoproteins. Based on their charge these compounds are almost certainly not partially hydrolysed phosphopeptides, but their nature is unknown.

Phosphoprotein Function. With the exception of the quartet o, p, q, and t, nothing is known about the intracellular localization of any of these proteins. In a recent study (6), we have observed that a group of phosphoproteins with the same M_r and isoelectric point as this quartet are found associated with fast-sedimenting chromosome complexes isolated from NH_4^+-starved cells. The similarity in phosphorylation phenotype and mobility on two-dimensional gels suggests that o, p, q, and t are nuclear phosphoproteins and are probably chromosome-associated. The recent finding that the start gene *CDC28* in yeast has significant structural homology to bovine cAMP-dependent protein kinase and a number of protein kinases encoded by oncogenes (13), as well as the evidence that the *CDC28* gene product has protein kinase activity (14) raises the interesting possibility that *CDC28* and other *cdc* start genes may encode protein kinases that phosphorylate some of the phosphoproteins described here. Since the *CDC28* nucleotide sequence suggests that the *CDC28* gene product is a substrate for a protein kinase (15), it is possible as well that some of these phosphoproteins may also be protein kinases and are part of a phosphorylation network transducing nutritional information to control the transition between proliferation and differentiation.

Conclusions. Under conditions of cell proliferation, when cAMP-dependent protein kinase is active, we find that a particular set of proteins (group 1) is phosphorylated. In arrested cells (arrest brought about either by inactivation of adenylate cyclase or NH_4^+ starvation), the phosphorylation of group 1 proteins is decreased, whereas that of another set of proteins (group 2) is increased. Group 2 proteins are not phosphorylated in *bcy1* cells even under NH_4^+-starvation conditions, while group 1 proteins remain phosphorylated under such conditions. This implies not only that group 1 proteins may be direct substrates for cAMP-dependent protein kinase, but that cAMP-dependent protein kinase is directly or indirectly responsible for negatively regulating the phosphorylation of group 2 proteins. The presence of group 1 proteins in *bcy1* cells under NH_4^+-starvation suggests that they may be necessary, but not sufficient, for proliferation. Group 2 proteins, phosphorylated on serine and threonine residues, appear when adenylate cyclase is inactivated, or in response to NH_4^+ starvation, suggesting that response to NH_4^+ is mediated in part by adenylate cyclase. A third class of phosphoproteins (group 3) are absent in *bcy1* cells but are present under proliferation and cell cycle arrest (either inactivation of adenylate cyclase or NH_4^+ starvation) conditions, revealing a difference between cells in which the activity of cAMP-dependent protein kinase is regulated and those in which it is not. Other phosphoproteins (group 4 and those in region K) whose phosphorylation can also be controlled independently of cAMP-dependent protein kinase have been identified.

There are recent indications that there may be more than one gene encoding catalytic subunits that can be regulated by cAMP (T. Toda, M. Zoller, and M. Wigler, personal communication). If this is true, then we cannot ascribe the phosphorylations observed in the presence of cAMP to a single enzyme. Since cAMP is likely to regulate all these enzymes in a similar fashion, this does not affect our conclusions, but it raises the question of which enzyme is instrumental in the negative regulation of group 2 and possibly group 3 protein phosphorylation.

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