Basal phosphorylation of cyclic AMP-regulated phosphoproteins in intact S49 mouse lymphoma cells

Robert A. STEINBERG* and Zoltan KISS†

Biological Sciences Group, U-44, University of Connecticut, Storrs, CT 06268, U.S.A.

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Protein phosphorylation in intact S49 mouse lymphoma cells was studied by using high-resolution two-dimensional gel electrophoresis of proteins labelled with [³⁵S]methionine or [³²P]P_i. In wild-type cells substrates for cyclic AMP-stimulatable phosphorylation exhibited high basal phosphorylation; in mutant cells deficient in activities of either cyclic AMP-dependent protein kinase or adenylate cyclase, basal phosphorylation of most of these substrates was negligible. Analysis of tryptic phosphopeptides from proteins labelled with [³²P]P_i in wild-type cells suggested that identical sites were phosphorylated under conditions of both basal and hormonally elevated concentrations of cyclic AMP. These results argue that most basal phosphorylation is a consequence of partial activation of cyclic AMP-dependent protein kinase and that this activation is attributable to basal concentrations of cyclic AMP. For the intermediate filament protein vimentin, basal phosphorylation was largely at a site distinct from that stimulated by increased cyclic AMP, and basal phosphorylation was not markedly different in mutant and wild-type cells. Vimentin phosphorylated at both sites was not observed. Cyclic AMP treatment resulted in enhanced phosphorylation at the cyclic AMP-specific site and decreased phosphorylation at the cyclic AMP-independent site.

A mechanism of considerable importance to hormonal regulation of mammalian cell functions is the cyclic AMP-regulated phosphorylation of specific substrate proteins. The central component of this regulatory system is cyclic AMP-dependent protein kinase (EC 2.7.1.37), an enzyme consisting of catalytic (C) and regulatory (R) subunits in an R₂C₂ complex. The tetrameric holoenzyme is catalytically inactive; binding of cyclic AMP to sites on R subunit promotes dissociation and concomitant activation of C subunit (Builder *et al.*, 1980). Enzymic activity also may be regulated by high-affinity protein inhibitors of C subunit that are found in many animal cells (Ashby & Walsh, 1972; Costa, 1977; Henry *et al.*, 1983). Consideration of kinetic parameters for kinase activation and of intracellular concentrations of kinase,

kinase inhibitor and cyclic AMP has led several groups to propose that activity of cyclic AMP-dependent protein kinase would be negligible under basal conditions (Beavo *et al.*, 1974; Ueland & Døskeland, 1976). ('Basal' is used to describe the condition of cells that are not hormonally stimulated. Empirically, basal cells are cells that have not been stimulated intentionally; possibilities for unintentional stimulation can rarely be eliminated absolutely.) Nevertheless many cyclic AMP-regulated phosphoproteins are phosphorylated to considerable extents under basal conditions both in cultured cells (Avruch *et al.*, 1978; Steinberg & Coffino, 1979; Groppi & Browning, 1980; Garrison & Wagner, 1982) and *in vivo* (Mayer & Krebs, 1970; Yeaman & Cohen, 1975; Sheorain *et al.*, 1982). The present studies were undertaken to determine whether basal phosphorylation was attributable to partial activation of cyclic AMP-dependent protein kinase or to the action of other cellular protein kinases with overlapping substrate specificities.

Assays of extracted protein kinase have not been very informative about the basal activation state of the enzyme. The ease with which kinase subunits

Abbreviations used: C and R subunits, catalytic and regulatory subunits of cyclic AMP-dependent protein kinase; SDS, sodium dodecyl sulphate; DME, Dulbecco's modified Eagle's medium.

* To whom reprint requests should be addressed.

† Permanent address: Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary.

can reassociate or dissociate during extraction and the different sensitivities of kinase isoenzymes to dissociation by salt and proteins make measurement of kinase activation unreliable at best (Corbin *et al.*, 1975; Ueland & Døskeland, 1976). Furthermore, the contribution of kinases unrelated to the cyclic AMP-dependent enzyme makes it essential to use the specific kinase inhibitor protein to distinguish basal activities contributed by C subunit and by other kinases. In cell-free extracts from a variety of tissues, basal kinase activities were about 20% of those revealed by addition of cyclic AMP (reviewed by Nimmo & Cohen, 1977), but these values were not corrected for activities of kinases other than C subunit. Our studies using the specific protein kinase inhibitor (Steinberg, 1981; R. A. Steinberg, unpublished work) or using mutants of S49 mouse lymphoma cells lacking C subunit activity (Steinberg *et al.*, 1978) suggest that most or all basal kinase activity in cell extracts is contributed by enzymes distinct from C subunit.

If different protein kinases were responsible for basal and cyclic AMP-stimulated protein phosphorylation, they might be expected to act at different sites. Comparisons of sites phosphorylated under basal and stimulated conditions have been made for only a few cyclic AMP-regulated phosphoproteins, and the results have varied between proteins. In rabbit skeletal muscle, phosphorylase kinase and glycogen synthase are phosphorylated at multiple sites; under basal conditions the cyclic AMP-dependent sites in phosphorylase kinase are negligibly phosphorylated (Yeaman & Cohen, 1975), but the cyclic AMP-dependent sites in glycogen synthase are phosphorylated to a considerable extent (Sheorain *et al.*, 1982). In isolated rat hepatocytes a 46000- M_r protein whose phosphorylation is stimulated by both insulin and glucagon yielded identical [32 P] P_i -labelled phosphopeptides whether labelled in the absence or presence of the hormones (Le Cam, 1982). Cyclic AMP-dependent phosphorylations in intact S49 mouse lymphoma cells have been characterized in several previous reports from our laboratory (Steinberg & Coffino, 1979; Steinberg, 1980, 1981). The present studies extend this work by assessing basal phosphorylation of cyclic AMP-regulated phosphoproteins in mutants deficient in either adenylate cyclase or cyclic AMP-dependent protein kinase and by examining tryptic phosphopeptides from proteins labelled under basal or stimulated conditions.

Experimental

Materials

Trypsin (type XI, diphenylcarbamoyl chloride-treated), DL-isoprenaline (isoproterenol) and 3-isobutyl-1-methylxanthine were from Sigma Chemical Co., St. Louis, MO, U.S.A.; [35 S]methionine (>900 Ci/mmol) was from Amersham Corp., Arlington Heights, IL, U.S.A.; and [32 P] P_i (carrier-free, in water) was from ICN Chemical and Radioisotope Division, Irvine, CA, U.S.A. Chemicals for two-dimensional gel electrophoresis were obtained as reported previously (Steinberg & Agard, 1981a,b).

Cells and media

S49 mouse lymphoma cells were grown in suspension culture in DME supplemented with 10% (v/v) heat-inactivated horse serum as described previously (Steinberg & Agard, 1981a). Wild-type cells were from subline 24.3.2, kinase-negative cells were from subline 24.6.1, and cyclase-negative cells were from subline 94.15.1 (Coffino *et al.*, 1975; Bourne *et al.*, 1975a; Steinberg *et al.*, 1978). Media for labelling experiments were prepared from 10-times concentrated DME lacking either methionine or phosphate and were supplemented with 10% heat-inactivated horse serum that had been dialysed extensively against 0.15 M-NaCl (Steinberg, 1983). Low-methionine DME contained 2.5 μ M-L-methionine, and low-phosphate DME contained 1 mM- P_i .

Radiolabelling and preparation of cell extracts

To label with [35 S]methionine, cells were centrifuged, washed once with low-methionine DME, and resuspended in low-methionine DME at a concentration of 2×10^7 /ml. After 5 min of preincubation at 37°C, cells were labelled for 12 min with 1 mCi of [35 S]methionine/ml. Incorporations were terminated by diluting cells 20-fold into 'conditioned media' prepared by filtration of media from harvested cell cultures (Steinberg, 1983). Where indicated, isoprenaline and isobutylmethylxanthine were included in chase media at final concentrations of 10 μ M and 50 μ M respectively. At the conclusion of chase periods, cells were centrifuged for 5 s at 10000g in a Fisher micro-centrifuge (Fisher Scientific Co., Medford, MA, U.S.A.), supernatants were aspirated, and cells were lysed immediately with gel sample buffer containing extra urea as described elsewhere (Steinberg, 1983). Media for both labelling and chase periods were equilibrated with CO₂ and prewarmed to 37°C to prevent drastic changes of temperature or pH; centrifugation and other manipulations of cell cultures were performed at room temperature.

To label them with [32 P] P_i , cells were washed and resuspended at a density of 2×10^6 /ml in low-phosphate DME. [32 P] P_i was added to a concentration of 0.5 mCi/ml, and cells were incubated for 3.5 h at 37°C. Where appropriate, isoprenaline (10 μ M) and isobutylmethylxanthine (50 μ M) were

added before harvest, as described in the Figure legends. Incorporations were terminated by diluting cultures 5-fold with ice-cold phosphate-buffered saline and harvesting and lysing cells as above.

Two-dimensional polyacrylamide-gel electrophoresis

The O'Farrell (1975) two-dimensional gel procedure was used with modifications described previously (Steinberg & Coffino, 1979). For all samples of a single experiment, first-dimension isoelectric-focusing gels were loaded with equal amounts of acid-precipitable radioactivity and run for 7000 V-h; second-dimension SDS-containing gels were 7.5% (w/v) in polyacrylamide. Labelled proteins were detected by direct autoradiography of dried gels by using Kodak XAR film (Eastman Kodak Co., Rochester, NY, U.S.A.). Gel patterns are shown with acidic proteins to the right.

Trypsin digestion and peptide analysis

Regions containing radiolabelled proteins were excised from dried gels by using tracings of autoradiograms as guides; gel pieces were hydrated,

and proteins were digested with trypsin as described previously (Steinberg & Agard, 1981b), except that a third portion of trypsin was added after overnight incubation and proteolysis was continued for another 7 h. After digestion, residual gel was removed, and soluble peptides were freeze-dried repeatedly to remove NH_4HCO_3 . Peptides were separated by t.l.c. on glass-backed cellulose plates (E. Merck, Darmstadt, W. Germany) in butan-1-ol/pyridine/acetic acid/water (15:10:3:12, by vol.) as described previously (Steinberg & Agard, 1981b). Phosphopeptides were detected by autoradiography at -70°C by using 'Lightning plus' intensifying screens (E. T. Du Pont de Nemours and Co., Wilmington, DE, U.S.A.).

Results

Fig. 1 illustrates the post-translational cyclic AMP-dependent phosphorylation responses of proteins from wild-type S49 mouse lymphoma cells. Cells labelled for 12 min with $[^{35}\text{S}]$ -methionine were diluted into medium without

Fig. 1. Two-dimensional electrophoretic analysis of cyclic AMP effects on $[^{35}\text{S}]$ methionine-labelled proteins in S49 mouse lymphoma cells

Wild-type S49 cells were labelled for 12 min with $[^{35}\text{S}]$ methionine, and then diluted into chase media without (a) or with isoprenaline plus isobutylmethylxanthine (b) and incubated for an additional 12 min. Samples containing 4×10^5 cells were harvested rapidly by centrifugation, and cells were lysed immediately with gel sample buffer. Portions of lysates containing 1.1×10^6 c.p.m. of acid-precipitable radioactivity were subjected to high-resolution two-dimensional gel electrophoresis; gels were dried and exposed to film for 18 days. Autoradiographic patterns shown are from regions of gels containing proteins with pI between about 5.7 and 6.8 and M_r values between about 40 000 and 140 000. Arrowheads indicate several proteins whose phosphorylation was stimulated by increased cyclic AMP. Letter designations are those used in previous papers (Steinberg & Coffino, 1979; Steinberg, 1981) except for 'T', which denotes a phosphoprotein not previously given a specific designation. For proteins A, I and P, both non-phosphorylated and phosphorylated forms are indicated by arrows; phosphorylated forms are to the right. For proteins M and T only phosphorylated forms are indicated.

(Fig. 1a) or with (Fig. 1b) isoprenaline, a potent activator of adenylate cyclase in these cells, and incubated for an additional 12 min before harvesting. (For all experiments described in this paper, isobutylmethylxanthine, a strong inhibitor of cyclic AMP phosphodiesterases, was used concomitantly with isoprenaline to enhance the increase in intracellular cyclic AMP.) Cells were lysed with a solution containing non-ionic detergent and saturated with urea to prevent post-lysis modifications, and the extracted proteins were separated by high-resolution two-dimensional gel electrophoresis. As we have reported previously (Steinberg & Coffino, 1979), phosphorylation could be detected by enhanced labelling of phosphoprotein species. For several phosphoproteins (e.g. those designated in Fig. 1 by the letters A and P), non-phosphorylated precursors were identified as slightly more basic species whose labelling was inversely related to that of the phosphoproteins. Phosphorylation of proteins A and P was essentially stoichiometric in the presence of high cyclic AMP concentrations, but phosphorylation of protein I, which has been identified as the intermediate filament protein vimentin (Steinberg & Coffino, 1979; Browning & Sanders, 1981), was limited to a small proportion of molecules labelled with [35 S]methionine. {Resolution of the two forms of [35 S]methionine-labelled vimentin was better in experiments described previously (Steinberg & Coffino, 1979; Steinberg, 1981).} From the labelling of endogenous cyclic AMP-stimulated phosphoproteins under basal conditions (Fig. 1a), it was apparent that significant phosphorylation had proceeded during the short labelling and chase periods; this basal phosphorylation was seen most clearly for proteins A and P, whose non-phosphorylated and phosphorylated forms were particularly well resolved. In similar experiments using longer chase periods, basal phosphorylation was still increasing after 30 min of chase and had reached an apparent steady state by 1–2 h of chase (results not shown).

Fig. 2 shows results from an experiment using [32 P] P_i labelling to compare basal phosphorylation in wild-type cells both with isoprenaline-stimulated phosphorylation in wild-type cells and with basal phosphorylation in cells lacking C subunit activity (kinase-negative cells). Wild-type (Figs. 2a and 2b) or kinase-negative (Fig. 2c) cells were labelled for 3.5 h with [32 P] P_i ; one portion of wild-type cells was treated with isoprenaline for the last 12 min of labelling (Fig. 2b). In wild-type cells the [32 P] P_i labelling of proteins M, P, I (vimentin) and T was stimulated by about 3-fold or more by isoprenaline. Basal labelling of protein P was much lower in kinase-negative than in wild-type cells. Basal labelling of proteins M and T also appeared

Fig. 2. Basal and cyclic AMP-stimulated [32 P] P_i labelling of substrate proteins in wild-type and kinase-negative S49 cells

Wild-type (a, b) or kinase-negative cells (c) were labelled for 3.5 h with [32 P] P_i before harvesting and preparing samples for electrophoresis. At 12 min before harvesting, isoprenaline and isobutylmethylxanthine were added to one portion of wild-type cells (b). Samples (6×10^6 acid-precipitable c.p.m.) of lysates were subjected to two-dimensional gel electrophoresis, and autoradiographs were exposed for 36 h. Arrowheads indicate positions of four of the phosphoprotein species shown in Fig. 1; letter designations are as in Fig. 1.

lower in kinase-negative cells, but the differences between wild-type and kinase-negative cells were less dramatic than for protein P. Basal phosphorylation of vimentin, on the other hand, was about the same in kinase-negative and in wild-type cells. As we have reported previously (Steinberg & Coffino, 1979), phosphorylated vimentin ran as a single species in two-dimensional gels for both basal and isoprenaline-stimulated cells. (Differ-

ences in labelling of other species in these patterns were not reproducible.)

To assess further the activities responsible for basal phosphorylations, we compared basal phosphorylation in wild-type S49 cells with that in kinase-negative and 'cyclase-negative' sublines. [Cyclase-negative cells are deficient in the nucleotide regulatory factor of adenylate cyclase (Johnson *et al.*, 1978).] Fig. 3 shows results from an experiment in which wild-type (Figs. 3a and 3b), kinase-negative (Figs. 3c and 3d), or cyclase-negative (Figs. 3e and 3f) cells were pulse-labelled with [35 S]methionine in low-methionine medium, then diluted 20-fold with conditioned medium containing normal concentrations of unlabelled methionine and incubated for an additional 4 h. Portions of autoradiographic patterns containing either protein A (Figs. 3a, 3c and 3e) or protein P (Figs. 3b, 3d and 3f) are shown. Although radioactivity in phosphorylated forms accounted for about 30–40% of the total A or P radioactivity in patterns from wild-type cells, it accounted for less than 10% of the radioactivity in these proteins from either type of mutant cell. Low basal phosphorylation was also observed in two additional cyclase-negative sublines that were derived independently (results not shown).

To determine whether or not basal phosphorylation involved the same sites as cyclic AMP-dependent phosphorylation, [32 P]P $_i$ -labelled species were excised from gels like those used to

Fig. 3. Basal phosphorylation of proteins A and P in wild-type, kinase-negative and cyclase-negative S49 cells. Wild-type (a, b), kinase-negative (c, d) or cyclase-negative cells (e, f) were labelled for 12 min with [35 S]methionine as for the experiment of Fig. 1, and then were diluted into chase media and incubated for an additional 4 h. Cells were harvested and lysed, and samples (1.3×10^6 acid-precipitable c.p.m.) from lysates were subjected to two-dimensional gel electrophoresis; autoradiographs were exposed for 18 days. Portions of gel patterns surrounding proteins A (a, c, e) and P (b, d, f) are shown. Arrowheads indicate positions of non-phosphorylated and phosphorylated forms of these proteins as in Fig. 1.

prepare the autoradiograms shown in Fig. 2 and subjected to digestion with trypsin. Fig. 4 shows autoradiographic patterns of thin-layer chromatograms of the resulting peptides for proteins P (Fig. 4a) and M (Fig. 4b). Radioactivity in digests from both proteins was mostly in one or two chromatographic species, but minor species were also seen (exaggerated in Fig. 4 by the long autoradiographic exposure used). For both proteins P and M the major tryptic phosphopeptides from basal and isoprenaline-stimulated cells were identical, thereby suggesting that phosphorylation was at the same sites. From kinase-negative cell preparations, little or no labelling of the major cyclic AMP-stimulated phosphopeptides was detected; contaminating phosphoproteins that overlap the positions of proteins P and M (see Fig. 2) probably account for the minor phosphopeptides present in about equal amounts in preparations from both wild-type and kinase-negative cells.

Fig. 5 shows tryptic phosphopeptides from vimentin labelled with [32 P]P $_i$ in wild-type (lanes 1–4) or kinase-negative cells (lane 5); the cells were either untreated (lanes 1, 5), or treated with isoprenaline for 5 (lane 2), 12 (lane 3) or 30 min (lane 4). A single major phosphopeptide species was intensified in chromatograms from the isoprenaline-treated cells, along with two minor species that probably represent incomplete digestion products containing the same site. A group of about four phosphopeptide species (also likely to represent a single phosphorylation site) were detected in digests of phosphorylated vimentin from kinase-negative cells. Since 'phosphorylated vimentin' ran as a discrete spot in gel patterns from kinase-negative cells, and since this region of the gel patterns was relatively free of other phosphorylated species, we presume that these four peptides derive from vimentin itself and not from co-migrating material. These phosphopeptides were also detected in the preparation from basal wild-type cells, but their amounts were markedly depressed in response to increased cyclic AMP. The isoprenaline-stimulated vimentin phosphopeptides were not detected in digests of phosphorylated vimentin from kinase-negative cells.

Discussion

The experiments presented in this paper allow us to draw four conclusions about basal phosphorylation of cyclic AMP-regulated phosphoproteins in S49 cells: (1) sites whose phosphorylation is stimulated by increased cyclic AMP are also generally phosphorylated under basal conditions; (2) basal phosphorylation of these sites depends largely, if not exclusively, on functional C subunits of cyclic AMP-dependent protein kinase; (3) the

Fig. 4. Tryptic phosphopeptides from proteins P and M

Wild-type and kinase-negative cells were labelled with [32 P]P_i in the presence or absence of isoprenaline and isobutylmethylxanthine as for the experiment of Fig. 2; lysates were prepared, and samples (10^7 c.p.m.) of acid-precipitable material were subjected to two-dimensional gel electrophoresis. Regions of gels containing proteins P (a) or M (b) were excised, and the proteins were digested exhaustively with trypsin as described in the text. Peptides were separated by t.l.c., and the resulting chromatogram was exposed to film for 13 days at -70°C with an intensifying screen for enhancement. Arrowheads indicate positions of major cyclic AMP-stimulated phosphopeptides; 'O' indicates the position of sample application. Lanes 1, 2 and 3 show, respectively, peptides from proteins labelled in basal wild-type cells, in wild-type cells treated with isoprenaline for the last 12 min of labelling, and in basal kinase-negative cells.

Fig. 5. Tryptic phosphopeptides from vimentin

Wild-type (lanes 1–4) and kinase-negative cells (lane 5) were labelled for 3.5 h with [32 P]P_i before harvesting and preparing samples for electrophoresis. Portions of the wild-type culture were treated with isoprenaline and isobutylmethylxanthine for 5 (lane 2), 12 (lane 3) or 30 min (lane 4) before harvesting. Samples (5×10^6 acid-precipitable c.p.m.) of cell lysates were subjected to two-dimensional gel electrophoresis; phosphorylated vimentin was excised from gels, and tryptic peptides were prepared and separated as for the experiment of Fig. 4. Autoradiography was for 16 days at -70°C with an intensifying screen. Arrowheads $\blacktriangleleft$ and $\triangleleft$ respectively indicate positions of peptides whose labelling was stimulated or decreased by increased cyclic AMP: $\blacktriangleleft$ indicates partial overlap of the two sorts of peptide. 'O' indicates the position of sample application.

extent of this basal phosphorylation is controlled by the activity of adenylate cyclase; and (4) where significant basal phosphorylation is observed in kinase-negative cells (e.g. in vimentin), it is at sites distinct from the cyclic AMP-regulated sites. Basal phosphorylation of cyclic AMP-regulated sites therefore appears to reflect partial activation of protein kinase by basal concentrations of intracellular cyclic AMP. Although S49 cells appear to contain protein kinase inhibitor in amounts sufficient to inactivate 10–15% of endogenous cyclic AMP-dependent protein kinase (Steinberg *et al.*, 1978), this activity does not prevent basal phosphorylation in intact cells.

Partial activation of the cyclic AMP response system in unstimulated wild-type cells might be expected to have effects on proteins whose synthesis is regulated by increased cyclic AMP as well as on those whose phosphorylation is under cyclic AMP control. This is apparently true for cyclic AMP phosphodiesterase and for kinase R subunit; both phosphodiesterase activity and R subunit synthesis are induced by increased cyclic AMP in wild-type cells, and their basal expression is decreased by about 2–3-fold in kinase-negative cells (Bourne *et al.*, 1975b; Steinberg *et al.*, 1979; Steinberg & Agard, 1981b). Since both phosphodiesterase and R subunit are negative regulators of the cyclic AMP response system, their sensitivities to kinase activation provide feedback mechanisms for fine-tuning the system. We have attempted to compare basal synthesis of other cyclic AMP-inducible proteins in wild-type and mutant cells, using analysis by two-dimensional gels of proteins labelled with [35 S]methionine, but synthesis rates were too low to assess reliably (R. A. Steinberg, unpublished work). Cyclic AMP also represses synthesis of several proteins in S49 cells (Steinberg & Coffino, 1979); basal synthesis of these proteins was apparently unaffected by kinase-negative or cyclase-negative mutations (R. A. Steinberg, unpublished work). This suggests that there may be differences in response parameters for different cyclic AMP-regulated functions. Such differences could explain why, despite the growth-inhibitory effect of cyclic AMP on wild-type S49 cells, kinase-negative and cyclase-negative mutations do not have dramatic or consistent effects on cellular growth rates (R. A. Steinberg, unpublished work).

The very low basal activity of adenylate cyclase in membranes from cyclase-negative cells (Bourne *et al.*, 1975a) suggests that basal concentrations of cyclic AMP should be decreased significantly in the mutants, but evidence supporting this proposition is unavailable. Since centrifugation and changes of temperature have been shown to have large, albeit transient, effects on cyclic AMP concentrations in lymphocytes (Moore *et al.*, 1983), we

worried that enhanced basal phosphorylation in wild-type cells might have resulted from artifactual stimulation of adenylate cyclase rather than from pre-existing differences in cyclic AMP concentrations. For the following reasons, we concluded that such an artifact was unlikely to have caused the observed differences between wild-type and cyclase-negative cells: changes in temperature or pH and centrifugation steps were avoided wherever possible in the present studies; basal phosphorylation in wild-type S49 cells was not transient; comparable steady-state extents of basal phosphorylation were observed under a variety of experimental protocols, including propranolol-mediated reversal of isoprenaline-stimulated phosphorylation (Steinberg, 1981); and R-subunit synthesis, which does not respond immediately to changes in cyclic AMP concentration (Steinberg & Agard, 1981b), was higher in basal wild-type than in basal cyclase-negative cells (R. A. Steinberg, unpublished work). It remains possible that some aspect of cell culture causes a persistent low-level activation of adenylate cyclase in wild-type cells. Serum contains high- M_r components that could cause such activation (Darfler *et al.*, 1984), but chronic exposure of S49 cells to serum actually decreases basal cyclic AMP concentrations (Darfler *et al.*, 1981). Whether or not extrinsic factors contribute to basal cyclase activity, the high basal phosphorylation of cyclic AMP-regulated phosphoproteins in most experimental systems suggests that such activity is common and that it has a significant effect on cellular metabolism.

For vimentin (protein I), less than 10% of molecules were modified even when kinase was maximally stimulated (Fig. 1; Steinberg & Coffino, 1979; Cabral & Gottesman, 1979; Gard & Lazarides, 1982a). Vimentin has sites for cyclic AMP-independent and cyclic AMP-dependent phosphorylation (Fig. 5; Gard & Lazarides, 1982b; Spruill *et al.*, 1983). Although multiply phosphorylated forms of vimentin have been reported by others (Gard & Lazarides, 1982b; Celis *et al.*, 1983), we could not detect significant labelling in the position expected for doubly phosphorylated vimentin (Fig. 2; Steinberg & Coffino, 1979; Steinberg, 1981; R. A. Steinberg & Z. Kiss, unpublished work). In our experiments dephosphorylation of the cyclic AMP-independent site was concomitant with phosphorylation of the cyclic AMP-dependent site; turnover of phosphate at the cyclic AMP-independent site, therefore, was rapid in the presence of cyclic AMP. Our failure to observe doubly phosphorylated vimentin may be explained by at least two different mechanisms: phosphorylation at one site might inhibit phosphorylation at a second site; or cyclic AMP might stimulate both phosphorylation and dephosphorylation of vimen-

tin by independent processes. In the former case, cyclic AMP-stimulated dephosphorylation of basal sites would be explained by competition between cyclic AMP-dependent and -independent kinases for a subpopulation of accessible vimentin molecules. Resolution among these and other possible models will require further characterization of phosphorylated and phosphorylatable vimentin.

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