Protein Kinases in HeLa Cells and in Human Cervix Carcinoma

W. Pyerin, N. Balbach, D. Kübler, and V. Kinzel

Institut für Experimentelle Pathologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-6900 Heidelberg

Z. Naturforsch. **36 c**, 552–561 (1981); received December 4, 1980/February 16, 1981

Protein Kinases, Cyclic AMP-Dependent and -Independent, HeLa Cells, Human Cervix Carcinoma, Histone, Phosvitin

Extracts of HeLa cell fractions were analyzed by DEAE- and phospho-cellulose chromatography for their range of cyclic AMP-dependent and -independent protein kinase activities phosphorylating histone and/or phosvitin; extractions were by phosphate buffered saline (soluble protein kinases) and the non-ionic detergent NP-40 (membrane-bound protein kinases). The soluble fraction contained (i) cyclic AMP-dependent histone kinases type I and II as evidenced by their behaviour on DEAE-cellulose and inhibition by the specific heat- and acid-stable protein kinase inhibitor (PKI) in a dose-related manner; both types I and II as well as their purified catalytic subunit also phosphorylated protamine and – with very low efficiency – casein but not phosvitin; (ii) a histone kinase (H), insensitive to cyclic AMP and PKI, also accepting protamine as substrate but not either casein or phosvitin; (iii) a phosvitin kinase (P), insensitive to cyclic AMP and PKI, which also phosphorylates casein but not histone or protamine. These four enzyme species were also found in NP-40 extracts of $27000 \times g$ residues which, however, contained further histone and phosvitin kinase activities as yet unspecified. NP-40 extracts of the microsomal fraction possessed, besides unspecified histone and phosvitin kinase activity, only the phosvitin kinase P and appeared to be devoid of histone kinases I, II, and H. The occurrence and ratios of the protein kinases classified suggest an ordered distribution over the diverse subcellular fractions of HeLa cells.

The overall pattern of soluble and membrane-bound histone and phosvitin kinases in extracts of cervix carcinoma tissue, the *in vivo* correlate of HeLa cells, closely resembled that of similar extracts of HeLa cells. HeLa cells hence appear, despite their long *in vitro* history, to express protein kinase activities similar to those of their *in vivo* ancestors, recommending them as a subject for the study of (certain) human protein kinase systems.

Introduction

The phosphorylation and dephosphorylation of proteins represents a powerful mechanism in the regulation of cellular processes. Prominent examples comprise processes within both the soluble and the membraneous parts of cells as well as their interplay (reviewed [1–4]), and phosphorylation has been implicated in the transforming activity of viruses causing leucaemias and sarcomas [5]. The protein kinases (EC 2.7.1.37, ATP: protein phosphotransferases) involved constitute several classes, some exhibiting cyclic nucleotide dependence while others do not.

Although a broad variety of animal tissues has been investigated for the presence of certain protein kinases, only a few data are available for human tissues. This has led us to investigate a human cervix carcinoma-derived cell line (HeLa) for its range of protein kinase activities phosphorylating histone and/or phosvitin which represent prototypes of basic and acidic substrates phosphorylated by functionally different forms of protein kinases (reviewed [4]). In order to establish their relevance to the *in vivo* situation, the protein kinase patterns observed were compared with those of a human cervix carcinoma. In addition, this study has enabled us to work out the basis for biochemical analysis of a protein kinase activity at the outer surface of HeLa cells which we reported recently [6]. The patterns were established on the basis of cell fractionation, differential extraction through phosphate buffered saline and the non-ionic detergent NP-40, and column chromatography (DEAE- and phospho-cellulose). For further characterization, cyclic AMP and the heat- and acid-stable inhibitor protein (PKI) specific for cyclic AMP-dependent protein kinases were employed as well as different substrate proteins.

Reprint requests to Dr. W. Pyerin.

0341-0382/81/0700-0552 $ 01.00/0

Materials and Methods

Histone (calf thymus; type II-A) and partially dephosphorylated phosvitin (egg vitellin) were obtained from Sigma, bovine serum albumin (cryst.,

100%), dithioerythritol (DTE), and N-morpholino-3-propane sulfonic acid (MOPS) from Biomol (Ilvesheim); adenosine cyclic 3′,5′-monophosphate (cyclic AMP) and protamine (salmon) were from Serva (Heidelberg), and Nonidet P-40 (NP-40) from Shell Chemicals. [γ-^{32}P]adenosine 5′-triphosphate (ATP; > 20 Ci/mmol) was purchased from New England Nuclear. All other chemicals used were from Merck and of the highest grade available.

Cloned *HeLa cells* were routinely cultivated as monolayers (plastic Petri dishes; 15 cm diameter; Falcon) in Minimum Essential Medium (MEM; Flow Laboratories, Irvine) containing Earle's salts supplemented with 10% calf serum and kept in an atmosphere of humidified air/CO_2 (95%/5%) at 37 °C. After reaching maximal density (5–8 days), the cells were harvested in phosphate buffered saline (pH 7.2) containing 2 mM ethylenediamine tetraacetate (EDTA). Pooled cells were washed two times with and finally suspended in phosphate buffered saline at a density of $3-4 \times 10^7$ cells/ml (Coulter counter; Coulter Electronics, Harpenden Herts). This suspension was either used immediately or kept at −70 °C until use. The cell line was free of mycoplasms as checked routinely (micro-biological assay, kindly performed by Dr. W. Nicklas).

Neoplastic cervix tissue excised from a 36 year old patient, was stored at −70 °C until use.

Extraction of protein kinase activities from complete HeLa cells, different HeLa cell fractions, and from cervix carcinoma tissue was as described below, all centrifugations at $27\,000 \times g$ being carried out for 20–30 min, and those at $105\,000 \times g$ for 90 min. *Complete HeLa cells* suspended in phosphate buffered saline at the density given above were mixed with NP-40 (final concentration 0.5%) and extracted for 15 min at 4 °C under occasional shaking. Nuclei and other particulate matter were removed by centrifugation at $27\,000 \times g$, and microsomes at $105\,000 \times g$; the supernatant (SN-105) of the latter was employed as extract. *HeLa cell fractions* were obtained from cell suspensions (as above) by differential centrifugation. For homogenization, suspensions were sonicated at 0 °C (Branson cell disrupter B 15; microtip, step 7; one sec-blasts at intervals of 20 sec) until microscopic inspection revealed > 95% destruction. As illustrated by the schemes included in Fig. 1, aliquots of homogenates were centrifuged at $27\,000 \times g$, the residue resuspended in phosphate buffered saline, sonicated again, and centrifuged as before. The last step was repeated, and the combined extracts (S-27) centrifuged at $105\,000 \times g$ resulting in a supernatant (S-105) containing the soluble protein kinase activities of HeLa cells. Membrane-bound protein kinases were extracted by NP-40 from residues of centrifugations at both $27\,000 \times g$ (R-27) and $105\,000 \times g$ (R-105). *R-27* was mixed with NP-40 in phosphate buffered saline to give a final detergent concentration of 0.1% or 0.5% (w/v), and extracted on ice several-fold supported by short sonications. Each step was finished by centrifugation at $27\,000 \times g$. The united extracts (SN-27) were finally centrifuged at $105\,000 \times g$, the supernatant (SN-105) representing the extract used in experiments. *R-105* was, prior to NP-40 extraction, treated 2–3 times by phosphate buffered saline/sonication/ultracentrifugation to release soluble protein kinases. Then NP-40 was added to a final concentration of 0.5% and sonication-supported extraction carried out on ice followed by $105\,000 \times g$ centrifugation. *Cervix carcinoma tissue* was minced with a surgical scalpel, suspended in phosphate buffered saline to give a ratio of wet weight/buffer of about 1/10, homogenized in an Ultra Turrax (type TP 18-10; Janke & Kunkel, Staufen) and subsequently in a glass-to-glass Potter-Elvehjem homogenizer. After filtration through a fine-meshed synthetic cloth and centrifugation at $4000 \times g$ for 30 min, the homogenate was mixed with NP-40 (final concentration 0.2%), briefly sonicated, and extracted overnight on ice. The extract was freed from particulate matter by centrifugation at $105\,000 \times g$.

DEAE-cellulose chromatography. Prior to chromatography, the respective extracts were dialyzed against buffer A (5 mM potassium phosphate, 2 mM EDTA, 1 mM DTE; pH 6.5) or buffer AN (buffer A/0.05% NP-40). Aliquots were applied to columns (Pharmacia K 9/15, or Wright GA 10 × 15) packed with DEAE-cellulose (DEAE-Sephacel; Pharmacia) and equilibrated with buffer A or AN depending on the buffer used for dialysis. The columns were washed with at least two bed volumes of buffer A or AN, and eluted with a gradient of NaCl in buffer A or AN (linear 0–300 mM NaCl, followed by 500 mM NaCl). 2–3 ml fractions were collected and assayed for protein kinase activity, protein, and ionic strength (conductivity; WTW-LF 42 apparatus, Weilheim).

Phospho-cellulose chromatography. Pooled DEAE eluates were dialyzed against buffer B (50 mM TRIS-Cl, 2 mM EDTA, 1 mM DTE, 0.5 M NaCl; pH 7.4) or

buffer BN (buffer B/0.05% NP-40). Aliquots were applied to columns (as above) packed with phospho-cellulose (cellulose phosphate, P-11; Whatman) and equilibrated with buffer B or BN depending on the buffer used for dialysis. Washing was with at least two bed volumes of buffer B or BN, and elution with a gradient of NaCl in buffer B or BN (linear 0.5–1.2 M NaCl, followed by 1.2 M NaCl). 2–3 ml fractions were collected and assayed for protein kinase activity, protein, and ionic strength (as above).

Catalytic subunit of cyclic AMP-dependent protein kinase type II was purified from HeLa cells according to the method developed for other mammalian tissues [7, 8].

Protein kinase activity was assayed at pH 6.8 in a final volume of 200 µl, the assay mixture comprising 10 µmol MOPS buffer, 2 µmol Mg-acetate, 25 nmol DTE, 1 nmol cyclic AMP (if appropriate), 2 nmol [γ-^{32}P]ATP, substrate protein (40 µg mixed histones or protamine, or 80 µg phosvitin or casein), and aliquots of preparations to be assayed for protein kinase activity. Preincubation for 3 min at 30 °C was followed by starting the reaction through addition of a mixture of substrate protein and [^{32}P]ATP; incubation lasted for 9 min at 30 °C. Termination of the reaction and removal of unspecifically bound ^{32}P was as described elsewhere [7] using, however, trichloracetic acid (TCA) at a concentration of 10%. Depending on the different problems encountered in the work presented, different types of control were employed: (i) incorporation of ^{32}P into endogeneous proteins, for which purpose BSA (80 µg/assay) was added instead of substrate proteins; (ii) incorporation of ^{32}P into substrate proteins in complete assay mixtures either at zero time, or at full length of incubation, where in the latter preparations to be assayed for protein kinase activity were replaced by heat-denatured preparations (80 °C; 15 min). One unit of enzyme activity was defined as that amount of enzyme which transfers 1 pmol phosphoryl group from ATP to recovered protein during 1 min at 30 °C. The data were not corrected for overall recovery of radioactive protein.

Protein determination was essentially by the method of Lowry *et al.* [9] using BSA as standard. Preparations containing DTE were treated with TCA (10%, v/v) two times and the precipitated protein determined.

Heat- and acid-stable protein kinase inhibitor (PKI) was purified from rat muscle as described elsewhere [10]. It was ensured that preparations did not possess any ATPase or phosphatase activity.

Results

Differential centrifugation and extraction

Disruption by sonication of HeLa cells suspended in phosphate buffered saline let to a protein kinase active homogenate (Fig. 1 A). After 27000 × *g* centrifugation, both the supernatant and the sediment displayed protein kinase activity. The latter could be freed extensively from buffer-extractable kinases due to resuspension, resonication, and repelleting; the supernatant of the second wash (S 3) usually contained less than 5% of the total protein kinase activity extracted (S-27), and, correspondingly, the residue (R-27) contained activity comparable to the residue of the preceding step.

The incorporation of ^{32}P into recovered protein was not significantly different in presence and absence of histone or phosvitin, either in homogenate or in any one of the fractions, indicating preferential phosphorylation of endogeneous proteins. The phosphorylation was not stimulated by cyclic AMP; the nucleotide had, if anything, an inhibitory effect.

Centrifugation of S-27 at 105000 × *g* resulted in a microsomal fraction (R-105) and a supernatant (S-105) both of which showing protein kinase activity. The total amount of protein kinase contained within the supernatant was defined as *soluble protein kinases*, thus including those present in the cytosol as well as those attached to membraneous elements in a form that can be stripped off by sonication and/or the ionic conditions employed. S-105 appeared to be the first fraction which phosphorylated both histone and phosvitin significantly better than endogeneous proteins; cyclic AMP stimulated phosphorylation of histone (1.1–2-fold), but seemed rather to inhibit that of phosvitin (by 10–60%).

The polyoxyethylene detergent NP-40 solubilized further protein kinase activities from residue R-27 as well as from residue R-105, both previously freed of soluble protein kinases. Kinase activities extracted in this way and remaining in the supernatant if centrifuged at 105000 × *g* (SN's-105), were defined as *membrane-bound.* (Centrifugation at this speed represents the crudest, but nevertheless essential criterion of any successful solubilization).

NP-40 extracts of R-27 (27000 × *g* residue) phosphorylated both histone and phosvitin by about 2-

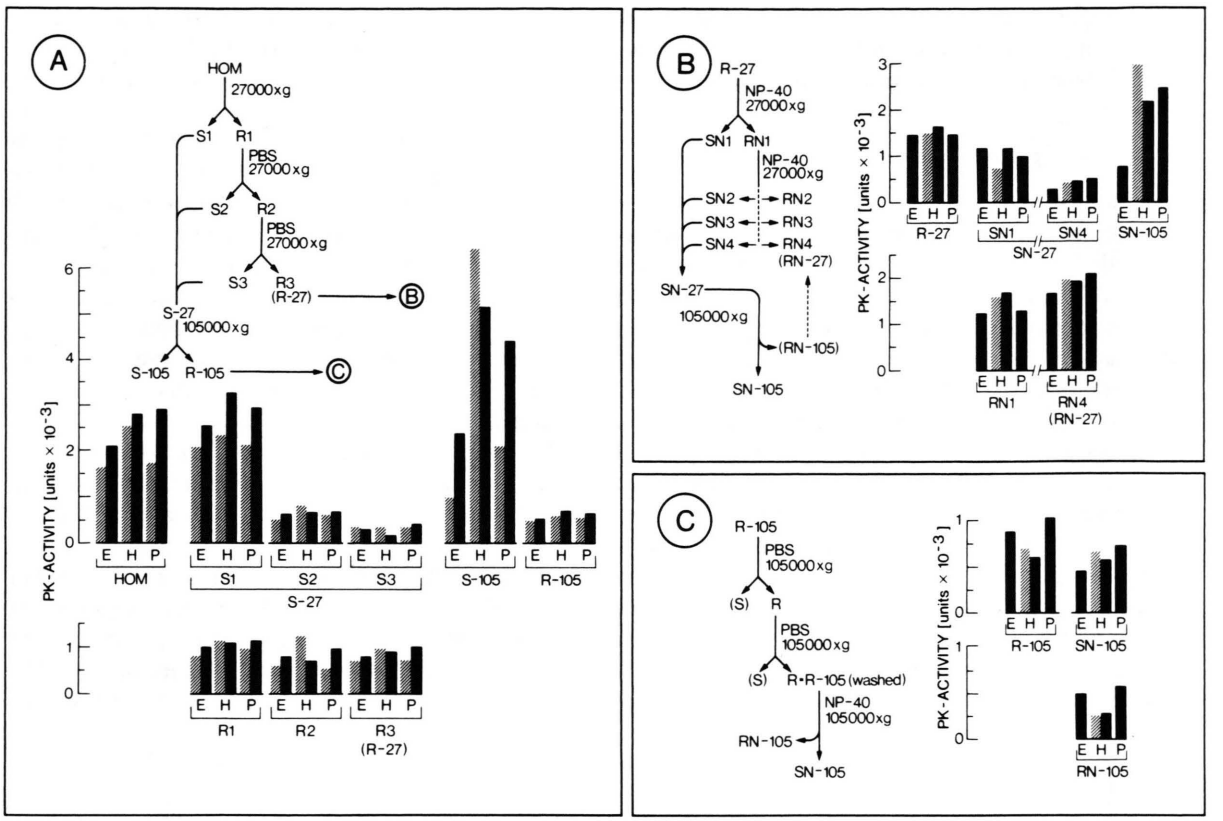

Fig. 1. Extraction of protein kinase activities from HeLa cell fractions. – *(A) Soluble fraction.* 2×10^8 HeLa cells were extracted in three steps through phosphate buffered saline/ultrasonication. After dialysis against buffer A, 25 µl aliquots of homogenate (HOM), supernatants (S1, S2, S3) and residues (R1, R2, R3) of the successive $27000 \times g$ centrifugations as well as supernatant (S-105) and residue (R-105) of $105000 \times g$ centrifugation were assayed in duplicate for protein kinase activity with histone (H) or phosvitin (P) as exogenous substrate, and for phosphorylation of endogenous proteins (E). All assays were carried out either in presence (hatched columns) or absence of 5 µM cyclic AMP. – *(B) Residue of 27000 × g centrifugation (R-27).* 3.2×10^8 HeLa cells were exhaustively extracted by phosphate buffered saline/ultrasonication and residue R-27 extracted by NP-40 at a final concentration of 0.1% in steps 1 through 3, and 0.5% in step 4. Extraction was for 20 min, the respective terminating $27000 \times g$ centrifugations leading to supernatants SN1–SN4 and residues RN1–RN4, and the following $105000 \times g$ centrifugation of the united extracts to supernatant SN-105. Protein kinase assays and symbols as in (A). – *(C) Microsomal fraction (R-105).* 6.7×10^8 HeLa cells were disrupted in phosphate buffered saline and microsomes pelleted. After extraction through phosphate buffered saline/ultrasonication (twice) and repelleting, NP-40 treatment was at a final concentration of 0.5% for 3 h, the following $105000 \times g$ centrifugation leading to supernatant SN-105 and residue RN-105. Assay for protein kinase activities and symbols as in (A).

fold better than endogeneous substrates, and cyclic AMP stimulated histone phosphorylation (Fig. 1 B). It is of interest to note that extraction did not deplete R-27, but on the contrary appeared to rather enhance its protein kinase activity; a significant increase along RN 1 through RN 4 was noted.

NP-40 extracts of R-105 (microsomal fraction) preferentially phosphorylated endogeneous proteins, addition of histone or phosvitin to assays only moderately increasing phosphoryl group transfer (Fig. 1 C). An influence on histone phosphorylation by cyclic AMP was not observed. If, however, unwashed microsomes were used, it did cause stimulation indicating that if cyclic AMP-dependent histone phosphorylation is found in this subcellular fraction, it obviously stems from cytosolic contaminants and/or sonication-sensitive attachments. Protein kinases which remained in the residue (RN-

105), prefered endogeneous proteins; addition of histone appeared to even inhibit phosphorylation both in presence and absence of cyclic AMP.

Separation of protein kinases

As shown in Fig. 2, chromatography on DEAE-cellulose of these different cellular fractions separated their protein kinase activities into several peaks. S-105, containing the soluble kinases, led to three main peaks (I, II, and III) on employment of a salt gradient (Fig. 2A). Very similar results have been obtained by chromatography of S-105 either directly or following ammonium sulfate precipitation at 60% saturation (see also [11]). Peak I and peak II activities eluted at about 50 mM and 200 mM NaCl, respectively. Both showed a strong cyclic AMP-dependent phosphorylation of histone; neither phosvitin nor endogeneous proteins were accepted as substrates. The I/II ratio might be approximated with 60/40; it changed somewhat from preparation to preparation. Peak III eluted at ≥ 300 mM NaCl. It contained only cyclic AMP-independent protein kinase activities; histone and phosvitin as well as endogeneous proteins were accepted as substrates.

Both the protein kinase pattern and the I/II ratio of the soluble fraction of HeLa cells has been confirmed in a report by Gray *et al.* [12] which appeared during preparation of this manuscript, if one assumes that the casein kinase shown in their paper represents the same enzyme as the peak III phosvitin kinase (see below); the peak III area, however, was not investigated for activities phosphorylating histone or endogeneous proteins, and most of the data were not established on isolated enzymes but rather indirectly by using crude extracts (27000 × *g* supernatants).

NP-40 extracts obtained from 27000 × *g* residue (R-27) similarly led to the three main peaks I, II, and III eluting at about 50, 200, and ≥ 300 mM NaCl, respectively; an extra peak (peak IV) was found at the front of the gradient (Fig. 2B). Peaks I and II showed both cyclic AMP-dependent histone phosphorylation, and the I/II ratio appeared to be shifted strongly towards type II reversing the I/II ratio seen in the soluble fraction. Histone was also

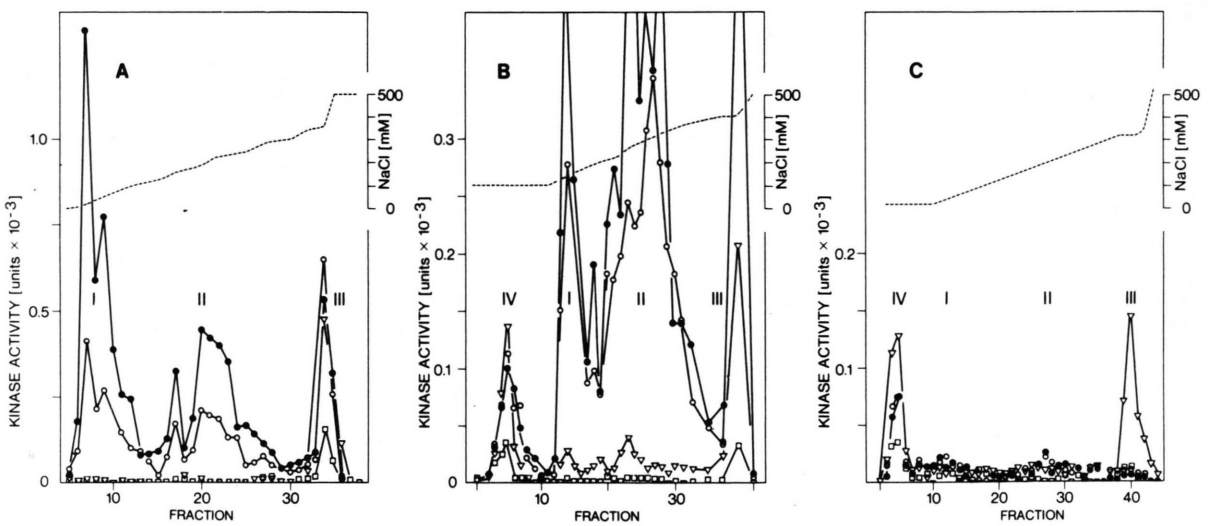

Fig. 2. DEAE-cellulose chromatography of HeLa cell extracts originating from different subcellular fractions. – *(A) Soluble fraction.* The S-105 fraction of 2×10^8 cells (10 ml) was dialyzed against buffer A and applied to a 0.9 × 13.5 cm DEAE column. Elution: NaCl gradient (- - -); 3 ml fractions. Assay of protein kinase activity with substrates: phosvitin (▽), histone (in presence (●) or absence (○) of 5 μM cyclic AMP), or endogeneous proteins (□). – *(B) NP-40 extract of 27000 × g residue (R-27).* Exhaustively extracted R-27 by phosphate buffered saline/sonication from 6.6×10^8 cells was extracted twice by NP-40 (final concentration 0.5%; first extraction for 3 h, second for 30 min) supported by short sonications (twice 3 pulses, 1/2 sec each). The SN-105 extracted (9 ml) was dialyzed against buffer A and applied to a 0.9 × 10.5 cm DEAE column. Elution, assays, and symbols as in (A). – *(C) NP-40 extract of microsomes (R-105).* R-105 pelleted from 1.5×10^9 cells was treated for three times by phosphate buffered saline/short sonication/ultracentrifugation and then extracted by NP-40 (final concentration 0.5%) for 3 h supported by a short sonication (once 3 pulses, 1/2 sec each). SN-105 (5.5 ml) was dialyzed against buffer AN and applied to a 1.0 × 10.4 cm DEAE column. Elution, assays, and symbols as in (A).

phosphorylated by peak III and IV activities but without stimulation by cyclic AMP. Phosvitin kinase activity was found again in peak III, but in contrast to S-105, also in all other peaks. Significant phosphorylation of endogeneous proteins was observed only in peaks III and IV. The peak IV activities were not influenced by PKI (for classification see below).

The microsomal extracts (SN-105) in contrast to the fractions above, were nearly devoid of histone kinases I and II; only some minor peaklets might be perceived (Fig. 2C). There was a peak at the gradient front (peak IV) showing phosvitin and cyclic AMP-independent histone phosphorylation as well as phosphoryl group transfer to endogeneous proteins. Another protein kinase peak elutes at ≥ 300 mM NaCl (peak III) displaying only phosvitin kinase activity.

Except peaks I and II of the soluble fraction, all kinase activities eluting from DEAE-columns were mixtures of histone and phosvitin kinases. Further separation was attempted by chromatography on phospho-cellulose.

While those mixtures constituting DEAE-peaks I, II, and IV eluted at front of the gradient and therefore remained unresolved (not shown), the mixture composing peak III was separated into a histone and a phosvitin kinase activity. As shown in Fig. 3, the histone kinase (H) eluted at the gradient front whereas the phosvitin kinase (P) was liberated from phospho-cellulose at 0.7–0.8 M NaCl. This result was obtained repeatedly with all the different extracts (cell fractions; complete cells) employed; as examples the profiles of the soluble fraction (Fig. 3A), the NP-40 extract of microsomes (Fig. 3B), and the NP-40 extract of complete cells (Fig. 3C) are shown.

Classification of isolated protein kinases

For classification of those histone and phosvitin kinases which eluted as single activities from DEAE- or phospho-cellulose columns, *i.e.* protein kinase I, II, H, and P, their behaviour was tested in presence of cyclic AMP and the heat-and acid-stable protein kinase inhibitor (PKI) specific for cyclic AMP-dependent histone kinases [4, 15]. Further their ability to phosphorylate protamine and casein.

Both peak I and peak II kinases were inhibited drastically by PKI in a positive dose-dependent manner (Fig. 4). Histone, protamine, and – with very low efficiency – casein were accepted as substrates, but not phosvitin (Table I). This provides evidence that these kinases represent in fact isoen-

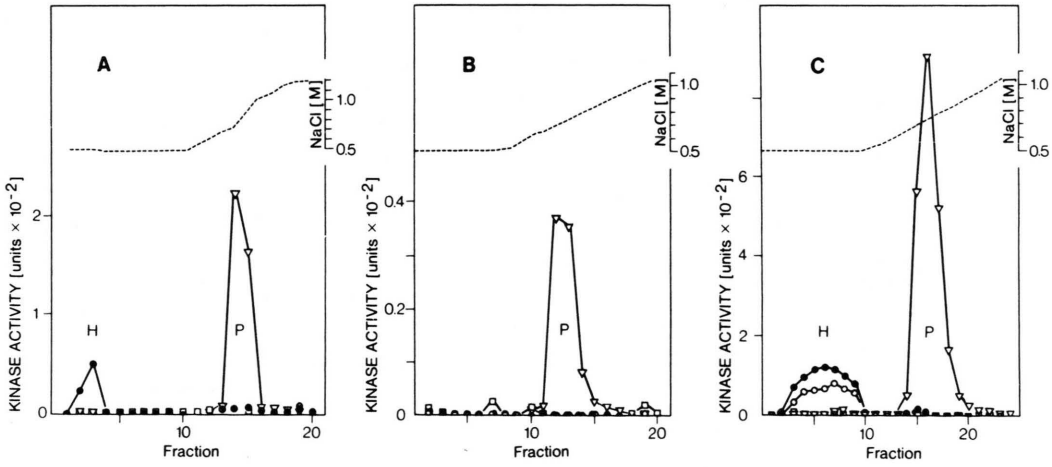

Fig. 3. Phospho-cellulose chromatography of protein kinase activity present in DEAE eluate peak III of extracts of HeLa cell fractions and complete cells. – *(A) Soluble fraction.* Pooled fractions of peak III/Fig. 2 A were dialyzed against buffer B and applied to a 0.9 × 9.8 cm phosphocellulose column. Elution: NaCl gradient (- - -), 3 ml fractions. Assay of protein kinase activity with substrates: phosvitin (▽), histone (●), or endogeneous proteins (□). – *(B) NP-40 extract of microsomes.* Pooled fractions of peak III/Fig. 2C were dialyzed against buffer BN and applied to a 0.9 × 9.8 cm phospho-cellulose column. Elution, assays and symbols as in (A). – *(C) NP-40 extracts of complete cells.* Pooled fractions of peak III/ Fig. 5A were dialyzed against buffer B and applied to a 1.0 × 10.2 cm phospho cellulose column. Elution, assays, and symbols as in (A).

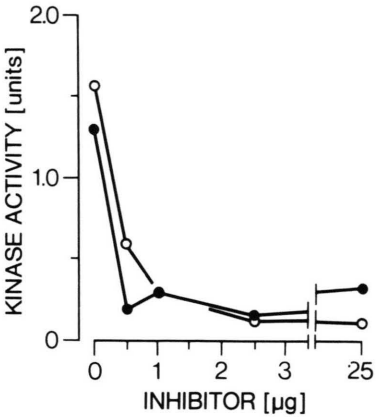

Fig. 4. Effect of heat- and acid-stable inhibitor protein on histone kinases I and II. 25 µl aliquots of histone kinase I (○) and II (●) after dialysis against buffer A was assayed for enzyme activity in presence of different amounts of PKI isolated from rat muscle. Histone kinases were tested in presence of 5 µM cyclic AMP.

zymes I and II of cyclic AMP-dependent protein kinases. The human cells under consideration, therefore, seem to be equipped with these enzymes in a similar fashion to a broad variety of mammalian tissues. These have been found to contain either nearly exclusively one or the other, or a more balanced mixture of both [7, 16].

In sharp contrast to isoenzymes I and II, the kinase activities gained as peaks H and P on phospho-cellulose were insensitive against the inhibiting action of PKI and remained uninfluenced by cyclic AMP (Table I). The histone kinase H accepted protamine as substrate with even higher efficiency than histone but neither casein nor phosvitin; the phosvitin kinase P also utilized casein with high efficiency but neither histone nor protamine. Obviously, the enzymes do not belong to the cyclic AMP-dependent category of protein kinases but to functionally different classes.

Comparison of the protein kinase patterns of complete HeLa cells and human cervix carcinoma

In order to find out whether those areas of expression concerning protein kinase activities were maintained in HeLa cells and hence still resemble the *in vivo* situation, a specimen of cervix carcinoma tissue was investigated for its protein kinase pattern and compared to that of HeLa cells. The cervix tissue was homogenized in phosphate buffered saline and further extracted under addition of NP-40 (final concentration 0.2%). The extract showed, after removal of particulate material at $105000 \times g$, cyclic AMP-dependent histone kinase activity (stimulation by the nucleotide was about 1.7-fold) and phosvitin kinase activity, both exceeding by far phosphorylation of endogeneous proteins. Chromatography on DEAE-cellulose and comparison of the pattern obtained with that of NP-40 extracts from complete HeLa cells showed that the two patterns possess regions of high similarity (Fig. 5). The HeLa cell extract – according to the specific properties of NP-40 [19] representing a mixture of soluble and predominantly nuclear-remote membraneous elements – led, as expected from the above results, to cyclic AMP-dependent (peaks I and II) and -independent histone kinases (peaks III and IV) as well as several phosvitin kinases eluting within regions I through IV (Fig. 3A). In close correspondence, the cervix sam-

Table I. Substrate specificity and effect of cyclic AMP and of heat- and acid-stable protein kinase inhibitor on isolated protein kinases of HeLa cells. Activities were determined in triplicate as in the standard assay in presence of the substances indicated.

Protein kinase	Effect of		Substrate phosphorylated			
	cyclic AMP [5 µM]	PKI[a]	Histone	Protamine	Casein	Phosvitin
I	+	+	+	n.d.[b]	n.d.	–
II	+	+	+	n.d.	n.d.	–
C subunit[c]	–	+	+	+	(+)	–
H	–	–	+	+	–	–
P	–	–	–	–	+	+

[a] Heat- and acid-stable protein kinase inhibitor; 26.7 µg added to assays, which inhibited 7.7 units of C subunit of cyclic AMP-dependent protein kinase activity by 98.8%, but had no influence on as little as 0.73 units of histone kinase H or 0.42 units of phosvitin kinase P.
[b] n.d. = not determined.
[c] isolated from type II histone kinase.

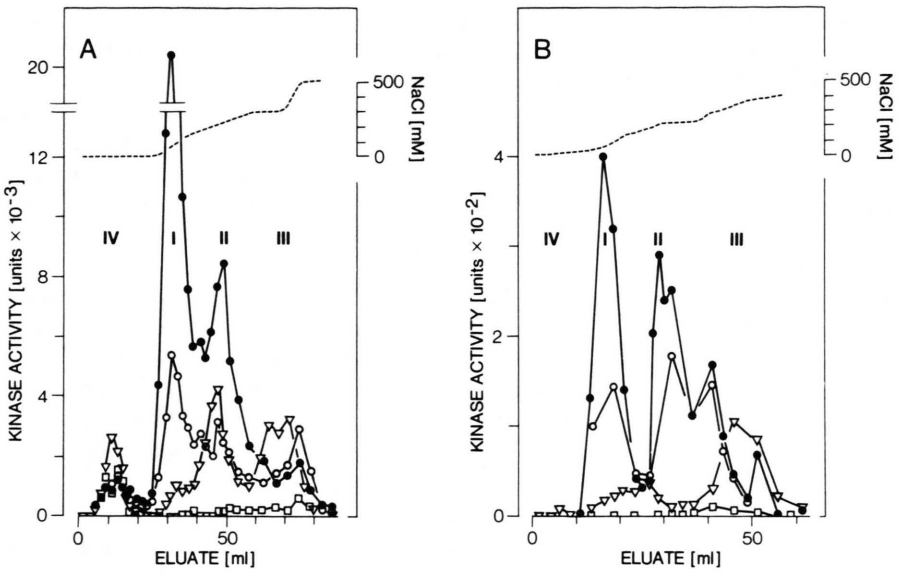

Fig. 5. DEAE-cellulose chromatography of NP-40 extracts from complete HeLa cells (A) and human cervix carcinoma tissue (B). *(A)* 1.9×10^8 HeLa cells were extracted by NP-40 (final concentration 0.5%) for 15 min and supernatant SN-105 dialyzed against buffer A. Chromatography of extract (8 ml) was carried out on a 1.0×10.2 cm DEAE column eluting with a NaCl gradient (- - -) in 2 ml fractions. Assay of protein kinase activity with substrates: phosvitin (▽), or histone in presence (●) or absence (○) of 5 μM cyclic AMP, or endogeneous proteins (□). *(B)* Homogenized cervix carcinoma tissue was extracted by NP-40 (final concentration 0.2%). Supernatant SN-105 (2.6 ml; equivalent of 160 mg tissue) was dialyzed against buffer AN and applied to a 0.9×9.5 cm DEAE column. Elution was carried out by a linear gradient of 0–400 mM NaCl (- - -). Assays and symbols as in (A).

ple extract (Fig. 3B) led also to peaks I, II, and III, displaying both histone and phosvitin kinase activities; kinase activity appeared to be missing only within the peak IV area.

Discussion

The existence of various protein kinases in many animal cells has been well documented and it seems little surprise that these enzymes are also present in cells of human origin. Indeed, certain human tissues and human cells in culture have already been shown to contain this sort of activity within their soluble parts (*e.g.* [13, 14]). Investigating soluble as well as membraneous elements, we show here that HeLa cells contain kinase activities able to phosphorylate both histone and phosvitin. The analysis carried out by DEAE- and phospho-cellulose chromatography revealed that each of this two prototypes of basic and acidic substrate proteins is phosphorylated by several different forms of kinases. Those which we were able to separate as single activities as yet, were identified as the cyclic AMP-dependent histone kinases type I and type II, a cyclic AMP-independent histone kinase H, and a cyclic AMP-independent phosvitin kinase P.

For classification, several parameters were used, since sensitivity to cyclic AMP alone is ambiguous even in connection with the chromatographic properties. Cyclic AMP-dependent protein kinases consist of regulatory (R) and catalytic (C) subunits; the nucleotide binds to R subunits and by that releases C subunits. These are insensitive to cyclic AMP and hence become undistinguishable from other cyclic AMP-independent histone kinases. Therefore, the ability to phosphorylate protamine and casein and the behaviour in presence of the heat- and acid-stable protein kinase inhibitor (PKI) have been used as further criteria. Since PKI specifically affects C subunits of cyclic AMP-dependent protein kinases and lacks tissue and species specificity [4, 15], it is especially valuable as a criterion of classification.

The kinase species identified are not distributed evenly over the whole cell but rather in a strictly ordered fashion. While phosvitin kinase P is present in every sub-cellular fraction, the other phosvitin kinases (contained within DEAE-peaks I, II, and IV)

are obviously bound to membraneous elements and released only by the aid of a detergent. Histone kinases I, II, and H are present predominantly in the soluble fraction and in particulate material sedimenting at 27000 × *g*. The I/II ratio, however, appears to be different. While type I predominates in the cytosol, type II predominates in the 27000 × *g*-residue. Microsomes, on the other hand, appear to lack these histone kinases or to contain them in a sonication-sensitive form only.

The pattern of distribution suggests that compartmentalization is an important factor for cellular processes controlled by these enzymes or for the control of their activity by the cell. However, this is by no means the only possibility involved in regulations of this kind. Among others, different modulating proteins of relatively small size are well known [10]. A hint to the complex interactions which may take place in HeLa cells is given by the observation that although kinase activity is removed from 27000 × *g* residue at each step of extraction by detergent NP-40, the remaining total activity appeared to rather increase. Although a variety of possibilities may be employed to explain this phenomenon – *e.g.* conformational changes due to NP-40, elevation of fluidity of membraneous structures and hence increased contact frequency of enzymes and substrates, abolished action or removal of inhibitors or of phosphoprotein phosphatases, etc. – experimental facts are lacking at the moment for reliable explanation.

The analysis of HeLa cell kinases was initiated to provide a basis for further and detailed studies of human protein phosphorylating systems. The relevance for the human situation of studies on HeLa cells, however, is questioned sometimes simply because activities found in these cells may not exist *in vivo*. This possibility, although unlikely, had to be considered carefully.

The original material for the cultivation of HeLa cells was human cervix carcinoma tissue (an epithelial tumor) withdrawn from a patient nearly 30 years ago [17]. HeLa cells, therefore, have a long *in vitro* history and some of the original cellular properties may indeed have been changed, although structural features characteristic for epithelial cells are still identifiable [18].

The comparative study presented here indicates that those areas of expression concerning protein kinase activities were maintained in HeLa cells and hence still resemble the *in vivo* situation. Using extracts of fresh cervix carcinoma tissue containing both soluble and membrane-bound kinases, the overwhelming majority of the different HeLa cell kinases were also found in the cervix tissue extract. Among them the cyclic AMP-dependent histone kinases type I and II, the cyclic AMP-independent histone kinase H, and the cyclic AMP-independent phosvitin kinase P.

The results strongly suggest that it would be worthwhile to study (at least certain) human protein kinase systems by the aid of HeLa cells and thereby fully utilize the advantages offered by cells in culture. A continuous supply of homogeneous material and the possibility of cell manipulation in particular are relatively unattainable with human tissue samples. The limited amount of clinical material usually available may then be used to verify (in a few experiments) whether the data obtained do in fact reflect the *in vivo* situation.

Acknowledgements

We thank Dr. M. Kaufmann for the carcinoma tissue sample and J. Richards for the cultivation of HeLa cells. The work has been supported by the Deutsche Forschungsgemeinschaft.

[1] R. A. Jungmann and D. H. Russell, Life Sci. **20,** 1787–1798 (1977).
[2] H. G. Nimmo and P. Cohen, Adv. Cyclic Nucleotide Res. **8,** 145–266 (1977).
[3] M. M. Hosey and M. Tao, Curr. Top. Membr. Transp. **9,** 233–320 (1977).
[4] M. Weller, Protein phosphorylation, Pion Ltd., London 1979.
[5] O. N. Witte, A. Dasgupta, and D. Baltimore, Nature **283,** 826–831 (1980).
[6] D. Kübler, W. Pyerin, and V. Kinzel, Hoppe-Seyler's Z. Physiol. Chem. **359,** 291–292 (1978).
[7] D. Kübler, M. Gagelmann, W. Pyerin, and V. Kinzel, Hoppe-Seyler's Z. Physiol. Chem. **360,** 1421–1431 (1979).
[8] W. Pyerin, M. Gagelmann, D. Kübler, and V. Kinzel, Z. Naturforsch. **34 c,** 1186–1194 (1979).
[9] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem. **193,** 265–275 (1951).
[10] M. Gagelmann, J. Reed, D. Kübler, W. Pyerin, and V. Kinzel, Proc. Nat. Acad. Sci. USA **77,** 2492–2496 (1980).
[11] J. L. Goldstein and M. A. Hasty, J. Biol. Chem. **248,** 6300–6307 (1973).

[12] J. P. Gray, R. A. Johnson, and D. L. Friedman, Arch. Biochem. Biophys. **202,** 259–276 (1980).
[13] J. C. Handschin and U. Eppenberger, FEBS Letters **106,** 301–304 (1979).
[14] K. P. Ray, T. B. Miller, and R. W. Butcher, Biochim. Biophys. Acta **584,** 181–195 (1979).
[15] J. A. Traugh, C. D. Ashby, and D. A. Walsh, Meth. Enzymol. **38,** 290–299 (1974).
[16] J. D. Corbin, S. L. Keely, and C. R. Park, J. Biol. Chem. **250,** 218–225 (1975).
[17] G. O. Gey, W. D. Coffman, and M. T. Kubicek, Cancer Res. **12,** 264–265 (1952).
[18] W. W. Franke, E. Schmid, K. Weber, and M. Osborn, Exp. Cell Res. **118,** 95–109 (1979).
[19] T. W. Borun, M. D. Scharff, and E. Robbins, Biochim. Biophys. Acta **149,** 302–304 (1967).