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Substrate-effected release of surface-located protein kinase from intact cells

(ecto enzyme/extracellular space/protein phosphorylation)

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ABSTRACT **Protein kinase activity that is independent of cAMP has been reported to exist on the surface of intact HeLa cells. Here we report that the protein kinase activity can be released by the use of casein or phosvitin within a short period of time. The discharge of the enzyme occurs from intact cells since (*i*) the cells do not release intracellular material and (*ii*) the cultures continue to grow without any morphological alteration. As shown with phosvitin, the release of protein kinase depends on substrate concentration, incubation time, and temperature. The degree of inducible release of surface protein kinase is inversely related to cell density. Four incubations with phosvitin (1 mg/ml) are sufficient to liberate most of the enzyme, thus greatly reducing the capacity of the cells to phosphorylate cellular substrates at the surface. Within approximately 24 hr after protein kinase removal, cultures have restored their surface protein kinase. Cultured cells of different origin (rat liver, mouse cerebellum, and human lung) exhibited phosvitin-induced protein kinase release from intact cells. The possible significance of these observations with respect to extracellular protein phosphorylation is discussed.**

In higher organisms, cells interact via their surfaces with the external environment of other cells, extracellular matrices, and fluids to form and maintain the multicellular state. The high degree of specificity necessary in these interactions has led to the idea that recognition of enzymes exposed at the cell surface by substrates may play a role (1–4) among possible communication mechanisms.

Intact HeLa cells carry protein kinase activity and substrates at their surfaces (5). A number of indications for the existence of surface-located protein kinase activity in other cell types have also been published (6–9).

Via surface-located protein kinase, HeLa cells are capable of interacting with their environment by phosphorylation of exogenous proteins, as shown by the use of phosvitin (5, 10). The potential for regulation through protein modification of this sort has been demonstrated in the case of plasma membrane receptors (11) and, more recently, for the transforming gene products of certain tumor viruses (12). Thus, suggestions of possible intercellular mechanisms demand closer investigation.

We have studied the possible release of surface protein kinase. Here we show that surface protein kinase activity can be selectively liberated from intact cells by the use of a substrate. These results suggest that externally directed protein phosphorylation represents an example of remote regulation occurring between separate cells.

MATERIALS AND METHODS

Radioisotopes were obtained from Amersham Buchler. Different batches of phosvitin, casein, histone, and reagents for determination of marker enzymes were purchased from Sigma; reagents for lactate dehydrogenase assay and cAMP were obtained from Boehringer Mannheim. Bovine serum albumin and trypan blue were obtained from Serva (Heidelberg, Federal Republic of Germany), trypsin ("1:250") was from Difco. Heat- and acid-stable inhibitor protein specific for catalytic subunits of cAMP-dependent protein kinases was isolated from rat skeletal muscle as described (13). ^{32}P-Labeled phosvitin was obtained by incubation of phosvitin (5 mg/ml) and [γ-^{32}P]ATP (0.5 μM; specific activity, 50 mCi/mmol; 1 Ci = 37 GBq) with intact HeLa cells for 15 min at 37°C. Cell supernatant was harvested and centrifuged at 2,500 × *g* for 10 min. Unbound [γ-^{32}P]ATP was removed from [^{32}P]phosvitin by gel filtration (1.5 × 5 cm column; Ultrogel AcA 202 from LKB). Gel electrophoresis and autoradiography showed that phosvitin from different batches may possess either one or two major substrate components as evident from Figs. 1 and 3.

HeLa cells were propagated in minimal essential medium/10% calf serum (Flow Laboratories) as described (5); embryo lung fibroblasts (HEL; human) were grown in basal medium Eagle's/10% fetal calf serum (Boehringer Mannheim). Hepatocytes from rat (provided by D. Mayer) were cultured in Ham's F12 medium/10% fetal calf serum; primary cultures from mouse cerebellum (provided by M. Schachner, Institute for Neurobiology, University of Heidelberg) were raised in basal medium Eagle's/10% horse serum (GIBCO Biocult). For experiments, cells were plated in plastic Petri dishes (Falcon; 3-, 5-, or 10-cm diameter). Synchronized HeLa cultures were obtained as described (14). Cell cycle traverse was monitored by flow cytometry (carried out by M. Stöhr) as described (5). Cells were propagated free from mycoplasms.

Preparation of cell supernatants was done at 37°C if not otherwise indicated. Cells were washed twice with prewarmed assay mixture (5 ml per 5-cm plate) consisting of 70 mM NaCl/30 mM Tris·HOAc/5 mM $Mg(OAc)_2$/5 mM potassium phosphate/0.5 mM EDTA/75 mM glucose, pH 7.2 (osmolarity, 290 ± 10 mOsm). Incubation was with 2 ml of assay mixture in the absence or presence of exogenous protein as given for the particular experiment. Cell incubation was done by moving the mixture gently over the cells (rocker platform; Bellco Glass) for various periods of time. Cell supernatants were centrifuged at 2,500 × *g* for 10 min and the flocculant was discarded. The remaining supernatant was kept in an ice bath until use. Control experiments in the absence of cells were carried out.

For phosphorylation of cell surface protein in the absence or presence of exogenous protein substrate, intact monolayer cells were treated as described for the preparation of cell supernatants but incubation was in the presence of 0.5 μM [γ-^{32}P]-ATP (5). The reaction was terminated after 10 or 15 min by sucking off the cell supernatant and washing the cells with two

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5-ml portions of ice-cold assay mixture containing 1 mM unlabeled ATP. Rinsed cells were immediately lysed with sample buffer (2.5% $NaDodSO_4$) and the lysate was subjected to $NaDodSO_4$ gel electrophoresis and autoradiography.

Protein kinase assay of cell supernatants was usually carried out in a total volume of 500 μl with fluid adjusted to a concentration of the particular protein substrate of 1 mg/ml. The reaction was started by addition of [γ-^{32}P]ATP (0.5 μM) and the mixture was kept in a shaking water bath at 30°C. For termination of the reaction, usually 100-μl aliquots were either spotted to chromatographic paper squares (which were immediately fixed in 20% trichloroacetic acid) or mixed with $NaDodSO_4$ solution. The material precipitated by trichloroacetic acid on chromatographic paper [2 × 2 cm; ET 31 (Whatman) for acidic protein or P 81 (Watman) for basic protein] was washed three times (30 min, 15 min, 15 min) with 20% trichloroacetic acid at 40–50°C to remove the remaining unbound labeled ATP as described by Goueli *et al.* (15). Radioactivity on the paper squares was determined in a scintillation counter (BF 5000/3; Berthold, Wildbad, Federal Republic of Germany). Protein was determined according to Lowry *et al.* (16) with bovine serum albumin as the standard.

Other enzymes were assayed as follows: lactate dehydrogenase (EC 1.1.1.27) according to Wroblewski and LaDue (17), Na^+/K^+-ATPase (EC 3.6.1.3) according to Blostein (18) and Crane (19), 5'-nucleotidase (EC 3.1.3.5) according to Ipata (20), succinate/cytochrome *c* reductase (EC 1.3.99.1) according to Avruch and Hoelzl Wallach (21), and NADH/cytochrome *c* reductase (EC 1.6.2.4) according to Sottocasa *et al.* (22). Phosphoprotein phosphatase activity was probed by using [^{32}P]phosvitin. Supernatants were incubated with [^{32}P]phosvitin (about 1.5 mg/ml) at 37°C in a shaking water bath for up to 20 min. Aliquots of the incubation mixture were analyzed for the remaining [^{32}P]phosvitin.

$NaDodSO_4$/polyacrylamide gel electrophoresis was carried out as described (5) in 7.5–15% polyacrylamide gradients. Radioactivity in gels was detected by autoradiography with x-ray film (Cronex 4 from DuPont or XR-5 from Kodak).

High-voltage paper electrophoresis was carried out at pH 3.5 and pH 2.0 with phosphoserine, phosphothreonine, and phosphotyrosine as references as given earlier (5).

RESULTS

Release of Protein Kinase Activity from Intact Cells. Monolayer HeLa cell cultures were incubated for 15 min at 37°C with the usual assay mixture (5) except for the absence of the protein substrate and ATP. Phosvitin and [γ-^{32}P]ATP were then added to the cell-free supernatant to assay protein kinase activity. Screening for radioactively labeled substrates was carried out by electrophoresis in the presence of $NaDodSO_4$ and autoradiography. As shown in Fig. 1, the supernatant from HeLa cells incubated in the absence of protein did not have detectable protein kinase activity. Very little was found if bovine serum albumin was present. However, if phosvitin or casein was included during incubation, appreciable phosphorylation was observed in the cell-free supernatant. Calf serum, which contains a specific substrate for surface protein kinase (5) also removes surface protein kinase. In contrast to the protein substrate, the cosubstrate ATP was unable to liberate protein kinase activity from HeLa cells.

Recently we pointed out the importance of selecting cytophilic substrates in working with cell surface protein kinase (10). Phosvitin and casein, in contrast to histones, do not release intracellular material such as lactate dehydrogenase and cAMP-dependent protein kinase from HeLa cells. No evidence was obtained for the release of microsomal or mitochondrial enzymes such as NADH/cytochrome *c* reductase and succinate/cytochrome *c* reductase by these substrates. Marker enzymes for the plasma membrane such as 5'-nucleotidase and Na^+/K^+-ATPase also were not detectable. Further, centrifugation at 105,000 × *g* of cell supernatants did not diminish protein kinase activity in the clear supernatant, suggesting that the enzyme released was not associated with the particulate fraction. In addition, earlier experiments have shown (10) that HeLa cells previously labeled with radioactive amino acids do not release radioactive material on treatment with phosvitin. HeLa cells continue to grow in the presence of phosvitin (5 mg/ml; ref. 5). The above results therefore indicate that phosvitin and casein selectively induce the release of protein kinase from intact cells. All further experiments were carried out with phosvitin.

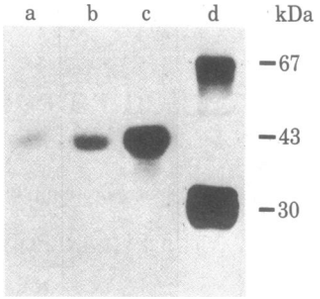

FIG. 1. Release of surface protein kinase from intact HeLa cells. Cells at 1 × 10^6 per dish were incubated for 15 min with assay mixture either without protein (lane a), or with serum albumin, phosvitin, or casein (all at 1 mg/ml) (lanes b–d) in the absence of extracellular ATP. After removal of the cell-free supernatants, phosvitin was added to the material shown in lanes a and b to 1 mg/ml, and it was further incubated with 0.5 μM [γ-^{32}P]ATP for 15 min. Aliquots from incubation mixtures were electrophoresed on $NaDodSO_4$/polyacrylamide gels and autoradiographed. kDa, Kilodaltons.

A time course of discharge of protein kinase activity from HeLa cells is shown in Fig. 2*A*. The values obtained (by the protein kinase assay carried out for 5 min) indicate that the re-

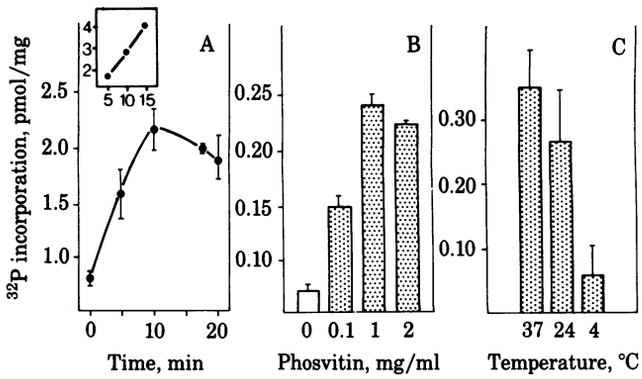

FIG. 2. Characteristics of the release process. (*A*) Time dependence. HeLa cells at 10^6 per dish were incubated with phosvitin at 1 mg/ml. At the times indicated, supernatants were harvested and the protein kinase assay was carried out for periods up to 15 min. Plotted are 5-min values. (*Inset*) Linearity of the assay up to 15 min with the supernatant harvested at 5 min. Results are for two cultures per group. (*B*) Substrate dependence. Cells at 0.5 × 10^6 per dish were treated with phosvitin at various concentrations for 5 min. Supernatants were removed and adjusted to a phosvitin concentration of 1 or 2 mg/ml of incubation mixture. ^{32}P transfer was determined for 5 min. Results are for three cultures per group. (*C*) Temperature dependence. Cells were treated with phosvitin as in *A* but at various temperatures. The protein kinase assay was carried out for 5 min. Results are for three cultures per group.

lease of protein kinase activity reaches a plateau after 10 min. The plateau may also occur earlier (data not shown). As shown in Fig. 2*A* (*Inset*) under the conditions used, phosphorylation progressed in each case linearly for at least 15 min; in all later experiments, phosphorylation was measured for 5 or 10 min. The release of protein kinase activity appeared dependent on the concentration of phosvitin in the supernatant (Fig. 2*B*) as well as on the temperature (Fig. 2*C*). The phosphorylation of phosvitin appeared independent of cAMP and was not inhibited by the heat- and acid-stable inhibitor specific for cAMP-dependent protein kinase. In either case, GTP could replace ATP although phosphorylation in the presence of GTP was less efficient (≈50%). The apparent K_m for ATP is $\approx 1 \times 10^{-6}$ M in the phosphorylation of phosvitin, which is the same order of magnitude as found for the phosphorylation of surface proteins (5). On partial acid hydrolysis of the phosphorylated protein substrates and high-voltage paper electrophoresis, seryl and threonyl residues were found to be phosphorylated (data not shown). Treatment of the supernatant with 0.06% trypsin solution for 10 min at 30°C abolished protein kinase activity. These properties are similar to those of surface protein kinase (5).

Disappearance and Restoration of Substrate-Inducible Protein Kinase Release. Even though the release of protein kinase activity reached a plateau by 10 min (Fig. 2*A*), it was apparently not complete (Fig. 3*A*). In this experiment, HeLa cells were incubated successively for four times, 5 min each, with fresh phosvitin solution (1 mg/ml). Autoradiographs showed that phosphorylation decreased with almost no activity in the fourth wash (lanes a–d). HeLa cells washed four times with phosvitin also completely lost their capacity to phosphorylate phosvitin directly (lane f). Four successive washes with phosvitin also reduced the capacity of HeLa cells to phosphorylate their own surface proteins as compared in lanes g and h. The cells separated in lanes g and h had been used to phosphorylate the phosvitin probes of lanes e and f, respectively. Because phosvitin competes with surface proteins for the label (5), the decrease of cell surface phosphorylation caused by prior treatment with exogenous substrate is even greater, particularly when the fact that the cells separated in lane h had all the [γ-^{32}P]ATP at their disposal for the phosphorylation of surface proteins is considered. The fact that phosvitin phosphorylated in the presence of cells was never found to be attached to these cells (e.g., lane g) indicates a very specific removal of the kinase. This is further evidenced by the experiment shown in Fig. 3*B*. Repetitive alternating incubation of cells in the absence and presence of phosvitin led to release of protein kinase only in the presence of substrate.

In the course of this study, we noticed that the protein kinase activity that could be released by phosvitin varied to some extent from experiment to experiment although it was reproducible within the same set of cultures. Therefore, the dependence of the induced enzyme release was analyzed in relation to cell number and cell density. A series of HeLa cell cultures having various numbers of cells was established over a 2-day period in Petri dishes of different sizes and analyzed for phosvitin-induced release of protein kinase activity. The different-sized culture plates were filled in with balanced volumes of phosvitin solution (1 mg/ml) as shown by Fig. 4. The data of three independent experiments (Fig. 4) indicated that the degree of inducible protein kinase release per cell decreased with increasing cell density. If protein kinase released from sparse cultures was mixed at different proportions with that of dense cultures, phosphorylation results were obtained as expected from the mixing ratios (data not shown). This excluded the possibility that the low degree of protein kinase activity recovered from dense cultures was a result of substrate, cosubstrate, or product

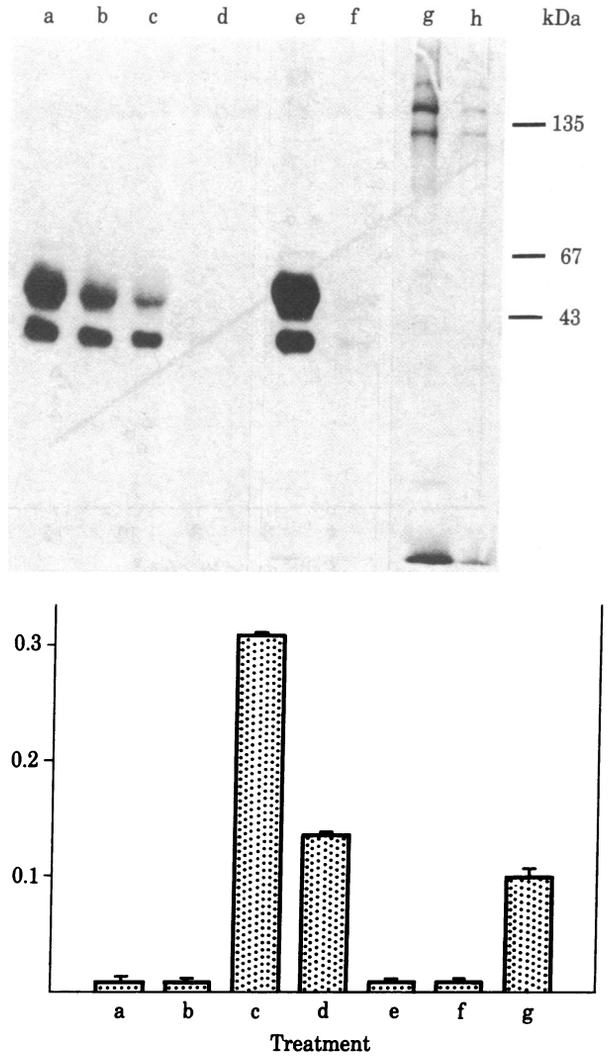

FIG. 3. (*A*) Depletion of surface protein kinase by iterative incubation with substrate. Cells at 1×10^6 per dish were incubated four times for 5 min each with fresh phosvitin at 1 mg/ml. Phosvitin phosphorylation was determined with 0.5 μM [γ-^{32}P]ATP for 5 min with cell-free supernatants (lanes a–d) or in the presence of cells before surface kinase release (lane e) or after treatment (lane f). Aliquots of the incubation mixture were electrophoresed and autoradiographed as in Fig. 1. [^{32}P]Phosphoprotein spectra of intact cells were obtained from fresh culture (lane g) or from surface enzyme-depleted culture after the fourth wash with phosvitin (lane h). kDa, Kilodaltons. (*B*) Substrate dependence of protein kinase release. Cells at 1×10^6 per dish were subjected to various treatments. Bars a and b: cells were washed with 2 ml of assay buffer in the absence of protein, and cell-free supernatants were obtained, treated with phosvitin to 1 mg/ml, incubated with [γ-^{32}P]ATP, and processed as in Fig. 2*A*. Bars c and d: cells were incubated with phosvitin at 1 mg/ml for 5 min, and cell-free supernatants were incubated with [γ-^{32}P]ATP and processed as above. Bars e and f: cells were washed with protein-free buffer, and cell-free supernatants were processed as described for bars a and b. Bar g: cells were incubated with phosvitin as for bar c, and cell-free supernatants were processed as for bars c and d. Results are for three cultures per group.

alteration (e.g., phosphoprotein phosphatases). The supernatant of HeLa cells incubated with [^{32}P]phosvitin was indeed shown to be free of phosvitin dephosphorylating capacity.

To study the reappearance of protein kinase sensitive to phosvitin-induced discharge, a series of HeLa cells was freed from most of the surface enzyme by two 5-min washes with phosvitin (1 mg/ml). The cells were further incubated in complete culture medium for different periods of time and ana-

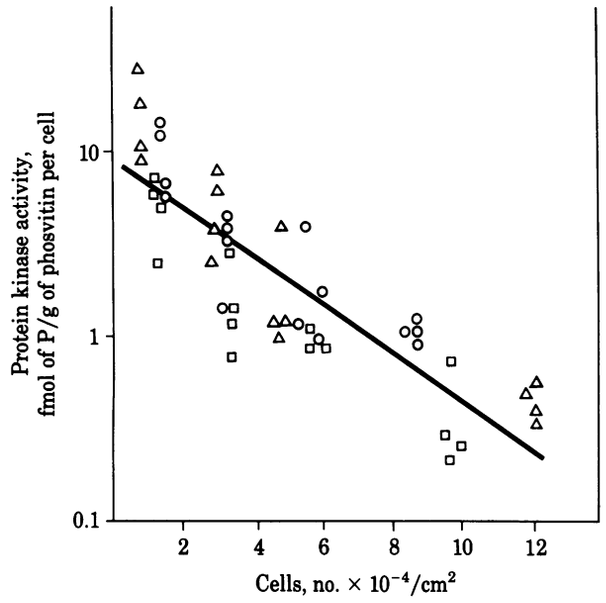

FIG. 4. Dependence of protein kinase release on cell number and cell density. HeLa cells grown in different sized plates to cell densities as indicated were incubated for 10 min with phosvitin at 1 mg/ml in various amounts of incubation fluid: ○, 0.7 ml in 3-cm plates; ▽, 2.0 ml in 5-cm plates; □, 7.8 ml in 10-cm plates. Protein kinase activity was determined for 5 min as described in Fig. 2*A*. Symbols represent values for single plates summarized from three independent experiments. The straight line was determined by linear regression from all values.

lyzed for inducible protein kinase release. Up to 7 hr, no recovery was observed. After 23–30 hr, releasable protein kinase had reappeared (Fig. 5*A*), albeit not to the original level probably because of the increase in cell density due to the doubling of the cell number during this time (Fig. 5*A Inset*). A similar result was obtained with HeLa cells synchronized for the beginning of S phase by amethopterin and freed of protein kinase

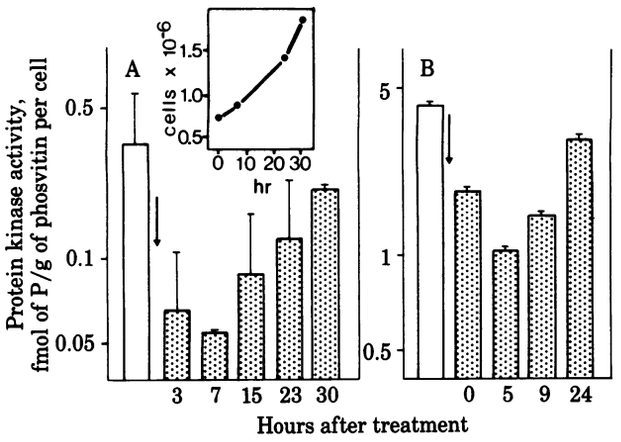

FIG. 5. Restoration of surface protein kinase release. (*A*) Reappearance in asynchronous cultures. HeLa cells at 0.7 × 10^6 per dish were freed from surface kinase by double treatment with sterile phosvitin at 1 mg/ml followed by incubation in minimal essential medium/10% calf serum under growth conditions. At the times indicated, three cultures were assayed for inducible protein kinase release (5 min). The empty column represents enzyme activity (0 hr) of cells not freed of surface kinase. (*Inset*) Cell multiplication during the experiment. (*B*) Reappearance in synchronized cultures. Cultures were synchronized by blockage with amethopterin. Surface enzyme was released at the time of removal of the blockage by addition of thymidine. Cells were then processed and the kinase assay was carried out as in *A*. Cell number was 0.5 × 10^6 at the time of release (0 hr) and 0.9 × 10^6 at the end of the experiment (24 hr). ↓, Kinase release.

at the time of release from cell cycle blockage (Fig. 5*B*).

Prevalence. Our observations led to the question of whether the inducible release of protein kinase activity represents a particular property of HeLa cells or is a more general property of cells, at least in tissue culture. Therefore, established cell lines and primary cultures were investigated under similar conditions. It was found that cells from rat liver parenchyme, mouse cerebellum, and human lung fibroblasts exhibited phosvitin-dependent release of surface protein kinase without showing any parameters of cellular damage.

DISCUSSION

Evidence is given for a rapid appearance of protein kinase activity in the supernatant of HeLa cells and other cells on treatment with the protein kinase substrate phosvitin. The enzyme belongs to the class of protein kinases independent of cyclic nucleotides that phosphorylate phosvitin and casein (5, 10) at seryl and threonyl residues. Because intact HeLa cells carry protein kinase activity at their surface (5) and also continue to grow in the presence of phosvitin, it was reasonable to assume that the release of protein kinase occurred from the intact cell. Moreover, the appearance of protein kinase in the supernatant was accompanied by concomitant diminution of surface-located protein kinase detectable on cells. That the protein kinase release occurs from intact cells is also supported by the failure to simultaneously detect intracellular or plasma membrane-derived enzymes in the supernatant. HeLa cells previously incubated for 44 hr with ^{14}C-labeled amino acids and treated with phosvitin did not release radioactivity (10). The amount of protein associated with any ^{14}C-labeled protein kinase discharged was probably too small to contribute detectable radioactivity. The recovery of protein kinase activity after induced release over approximately 24 hr also argues for its origin from intact cells, which also supports the observation that surface-located protein kinase is not derived from damaged cells (5, 10).

The phosvitin-induced discharge of surface protein kinase differs basically from exocytosis by the fact that, at the time of the stimulation, protein kinase is already exposed at the surface. The induced discharge is also dissimilar from spontaneous shedding as this process represents a comparably slow release of surface material (23). The temperature dependence of protein kinase discharge, however, is reminiscent of the release of T-cell receptors stimulated by alloantigen (24).

Without phosvitin, spontaneous release of protein kinase was scarcely detectable within the time frame of the experiments; very little enzyme was released in the presence of bovine serum albumin. Phosvitin seems to cause a selective discharge of protein kinase that is probably not due to random association with the cell surface, since no phosphorylated phosvitin was found together with the cells after two washes with isosmotic solution. It seems likely that the substrate releases protein kinase by direct interaction with the enzyme. At present, it is not clear why doubling the phosvitin concentration from 1 to 2 mg/ml does not increase the release of protein kinase during 5 min whereas a second wash at 1 mg/ml yields additional protein kinase in the supernatant. These observations may partially explain why the continued presence of calf serum, which contains a specific substrate (5), does not deplete the cells of surface protein kinase.

The nature of the association of the enzyme with the surface and the mechanism of how phosvitin overcomes the forces that keep the enzyme bound remain to be analyzed. Evidence collected so far seems to argue against a proteolytic activity introduced by phosvitin. Kinetic measurements with highly purified phosvitin kinase (unpublished data) and the linearity of

the phosphorylation of phosvitin by released protein kinase seem to exclude such contamination. Furthermore, discharge of protein kinase should be impaired in the presence of 10% calf serum, which is known to contain a number of protease inhibitors (25), but this was not the case. It may be possible that, on binding to the enzyme, phosvitin causes a conformational change that leads to discharge of the enzyme from the membrane. The anchorage of surface protein kinase by binding to cellular substrates of the cell surface seems to be unlikely since ATP is unable to induce a release.

Whereas the discharge of surface protein kinase seems to follow an immediate mechanism, the reappearance of the enzyme is comparatively slow—probably during membrane turnover and as a result of the increase of the cell number. That dense cultures discharge relatively less protein kinase per cell than sparse cultures on treatment with phosvitin could result from decreased accessibility or masking of the enzyme. Indications for an inverse relationship between cell density and release of surface material were also obtained by Ramseier (24).

Data pointing to the existence of surface-located protein kinase have been obtained from a variety of cells (6–9, 26). The only indication published to our knowledge that phosvitin may release protein kinase from cells was interpreted as a result of cell leakiness (27), which was excluded in our studies. It was therefore not surprising that all cultured cells studied were able to release the enzyme on treatment with phosvitin.

To understand the physiological significance of protein kinase in this location, one needs (*i*) to compare the degree of protein kinase discharge from different cell types systematically in relation to their biology and (*ii*) to search for and analyze natural substrates and the significance of their phosphorylation. As to the availability of cosubstrate ATP for cell surface protein kinase, there is positive support from measurements of the ATP level in extracellular fluids (28, 29). Such ATP might derive from translocation of cytoplasmic ATP into extracellular space (1), formation of extracellular ATP involving enzymes on the external surface (30), or cytolysis of dying or damaged cells (29). With regard to the physiology, it will be necessary to determine whether the phenomena of surface-bound protein kinase and extracellular free protein kinase are results of the artificial tissue culture situation or whether they reflect properties of cells situated in the organism. A preliminary study (26) has indicated that in several body fluids other than blood plasma or serum extracellular phosvitin kinase is present.

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1. Trams, E. G. (1974) *Nature (London)* **252,** 480–482.
2. Shur, B. D. & Roth, S. (1975) *Biochim. Biophys. Acta* **415,** 473–512.
3. DePierre, J. W. & Karnovsky, M. L. (1973) *J. Cell Biol.* **56,** 275–303.
4. Bosmann, H. B. (1977) *Int. Rev. Cytol.* **50,** 1–23.
5. Kübler, D., Pyerin, W. & Kinzel, V. (1982) *J. Biol. Chem.* **257,** 322–329.
6. Mastro, A. M. & Rozengurt, E. (1976) *J. Biol. Chem.* **251,** 7899–7906.
7. Ronquist, G., Ågren, G., Eklund, S. & Wernstedt, C. (1977) *Upsala J. Med. Sci.* **82,** 1–5.
8. Kang, E. S., Gates, R. E., Chiang, T. M. & Kang, A. H. (1979) *Biochem. Biophys. Res. Commun.* **86,** 769–778.
9. Sommarin, M., Henriksson, T. & Jergil, B. (1981) *FEBS Lett.* **127,** 285–289.
10. Kübler, D., Pyerin, W. & Kinzel, V. (1982) *Eur. J. Cell Biol.* **26,** 306–309.
11. Carpenter, G., King, L., Jr., & Cohen, S. (1979) *J. Biol. Chem.* **254,** 4884–4891.
12. Barnekow, A., Boschek, C. B., Zimiecki, A., Friis, R. R. & Bauer, H. (1982) in *Genes and Tumor Genes,* eds. Winnacker, E. & Schoene, H. H. (Raven, New York), pp. 65–73.
13. Walsh, D. A., Ashby, C. D., Gonzalez, C., Calkins, D., Fischer, E. H. & Krebs, E. G. (1971) *J. Biol. Chem.* **246,** 1977–1985.
14. Mueller, G. C. & Kajiwara, K. (1969) in *Fundamental Techniques in Virology,* eds. Habel, K. & Salzman, N. P. (Academic, New York), pp. 21–27.
15. Goueli, S. A., Slundgard, R., Wilson, M. J. & Ahmed, K. (1980) *J. Pharmacol. Methods* **3,** 235–242.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
17. Wroblewski, F. & LaDue, J. S. (1955) *Proc. Soc. Exp. Biol. Med.* **90,** 210–213.
18. Blostein, R. (1968) *J. Biol. Chem.* **243,** 1957–1965.
19. Crane, R. K. & Lipmann, F. (1953) *J. Biol. Chem.* **201,** 235–243.
20. Ipata, P. L. (1967) *Anal. Biochem.* **20,** 30–36.
21. Avruch, J. & Hoelzl Wallach, D. F. (1971) *Biochim. Biophys. Acta* **233,** 334–347.
22. Sottocasa, G. L., Kuylenstierna, B., Ernster, L. & Bergstrand, H. (1967) *J. Cell Biol.* **32,** 415–438.
23. Black, P. H. (1980) *Adv. Cancer Res.* **32,** 75–199.
24. Ramseier, H. (1974) *J. Exp. Medicine* **140,** 603–618.
25. Heimburger, H. (1975) in *Proteases and Biological Control,* Cold Spring Harbor Conferences on Cell Proliferation, eds. Reich, E., Rifkin, D. B. & Shaw, E. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 367–386.
26. Kübler, D., Pyerin, W., Burow, E., Garçon, T. & Kinzel, V. (1982) in *Cell Function and Differentiation,* Part A, Proceedings of the Special FEBS Meeting on Cell Function and Differentiation, eds. Akoyunoglou, G., Evangelopoulos, J., Georgatsos, J., Palaiologos, G., Trakatellis, A. & Tsiganos, C. P. (Liss, New York), pp. 499–509.
27. Chiang, T. M., Kang, E. S. & Kang, A. H. (1979) *Arch. Biochem. Biophys.* **195,** 518–525.
28. Bishop, C., Rankine, D. M. & Talbott, J. H. (1959) *J. Biol. Chem.* **234,** 1233–1237.
29. Trams, E. G., Kaufmann, H. & Burnstock, G. (1980) *J. Theor. Biol.* **87,** 609–621.
30. Ågren, G. & Ronquist, G. (1969) *Acta Physiol. Scand.* **75,** 124–128.