Protein Kinase Activity and Substrates at the Surface of Intact HeLa Cells*

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Evidence is presented for the location at the surface of HeLa cells of a protein kinase capable of phosphorylating surface as well as extracellular (foreign) proteins. The reaction products have been found to be proteins containing phosphoryl groups as monoesters of seryl and threonyl residues (but not of tyrosine). The enzyme is of the cyclic AMP-independent type, since neither cyclic AMP nor the heat- and acid-stable inhibitor protein (specific for cyclic AMP-dependent protein kinases) influenced its activity. Further, co-substrate ATP could in part be substituted by GTP, and the spectrum of proteins phosphorylated by the ecto-enzyme differed from that phosphorylated by cyclic AMP-dependent protein kinases. Evidence for the ecto-enzymic nature of this protein kinase includes (a) utilization of co-substrate and location of products at the surface of cells carefully controlled as being in an intact state and (b) phosphorylation of exogenous protein (phosvitin; specific serum proteins) by intact cells. Conclusive proof was gained by qualitative and quantitative comparative studies of phosphorylation in cultures with varying degrees of damaged cells either as a whole or after separation into groups of intact and damaged cells by electronic cell sorting. The results of experiments with cell sonicates excluded the possibility that either enzyme or substrates released from damaged cells were simply adsorbing to the cell surface.

In multicellular organisms, the cell surface seems to play an important role in growth control, differentiation, and cell-cell communication. Enzymes of the cell surface may be involved (3, 9, 40). Protein kinases (EC 2.7.1.37) in particular would be likely candidates for mediating some of the observed phenomena since phosphorylation is known to alter structure and function of proteins in a reversible manner (12, 13, 45).

Plasma membranes isolated from eukaryotic cells contain a variety of protein kinases as well as their substrates (13, 20, 38). Some of these have been localized on the inner side of the membrane (46) including the recently discovered src gene protein kinase (7). However, a few extensive attempts have been made to attribute protein kinase activity to the outer surface of cells (15, 26, 29, 34, 39). The major problem is that such studies have to be carried out with intact cells, since on perturbation or damage of the membrane barrier, intracellular enzymes (and substrates) become accessible and mask any ecto-kinase activity because of their high excess. For this reason, the degree of interference of intracellular protein kinase in the reaction measured has to be carefully controlled. Unfortunately, this has not been done in the studies made to date. On the contrary, in some studies even histones, which have been found to be cytotoxic and/or cause leakage in a variety of cells such as Dictyostelium (33), HeLa, 3T3, and SV 3T3 cells, have been utilized in order to demonstrate surface protein kinase, in our opinion a self-defeating approach.

In this paper evidence is given for ecto-protein kinase activity on intact HeLa cells which catalyzes for the phosphorylation of cell surface as well as of exogenous proteins. That the kinase activity observed is in fact associated with the outer membrane of the cell is demonstrated first, through localization of the products of phosphorylation at the cell surface, and second, through its characteristic interaction with exogenous substrates and co-substrates. In all cases, rigorous controls were employed to ensure that the cells were intact.

EXPERIMENTAL PROCEDURES

Materials—Histones (calf thymus, catalogue No. H 9250) and phosvitin (egg vitellin) were purchased from Sigma (Munich). Bovine serum albumin, reagents for polyacrylamide gels, nucleases (DNase I, RNase A), and 4\(^{-}\)-diamidino-2-phenylindol-2HCl were obtained from Serva (Heidelberg). The N\(^{6}\),2'-O-dibutylryl derivative of cAMP (Bt\(_2\)-cAMP) and the reagents for determination of lactate dehydrogenase activity were obtained from Boehringer-Mannheim and tryptic ("1:250") from Difco (Detroit). [\(\gamma\)-32P]ATP (>20 Ci/mmol), 32P-orthophosphoric acid (carrier free), 32P-pyrophosphate (100 to 400 mCi/mmol), and [\(\gamma\)-32P]gTP (20 Ci/mmol) were from New England Nuclear (Boston), and U-\(^{14}\)C-labeled protein hydrolysate (>80 mCi/mmol) from Amersham Buchler (Hannover). All other reagents used were of analytical grade. The heat- and acid-stable inhibitor protein specifically acting on CAMP-dependent protein kinase was isolated from rat muscle tissue according to the procedure given by Walsh et al. (44).

Cell Cultures—Cloned HeLa cells (human) were cultured routinely as monolayers in Minimum Essential Medium (MEM) containing Earle's salts supplemented with 10% (v/v) calf serum (Flow Laboratories, Irvine) and kept in humidified air/CO\(_2\) (95:5; 37° C) atmosphere. The cell line was free from mycoplasms (microbiological assays kindly performed by Dr. W. Nicklas, German Cancer Research Center). The condition of the cell culture in terms of cell viability was checked by trypan blue exclusion (0.1% in phosphate-buffered saline; Serva, Heidelberg) and determination of fluorescent cells after staining with DAPI. Cultures with purposely higher levels of damaged cells were obtained by placing approximately 10\(^6\) cells on 60-mm plate followed by incubation for 3 to 7 days without change of medium. For preparation of sonicates, cells were scraped off the culture dish in ice-cold assay mixture (see below). The suspension was sonicated at 0° C (Branson cell disruptor B 15, microtip, step 3; 5-s blasts with

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2. The abbreviations used are: Bt\(_2\)-cAMP, N\(^{6}\),2'-O-dibutylryl-cAMP; BSA, bovine serum albumin; cAMP, cyclic adenosine 3',5'-monophosphate; DAPI, 4',6-diamidino-2-phenylindol-2HCl; EDTA, ethylene-diaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.
10-s intervals) for 2 min and centrifuged at 4000 × g for 10 min. The supernatant was kept ice-cold until used.

Phosphorylation Conditions—For routine phosphorylation the following conditions were chosen. HeLa cells were washed twice with prewarmed assay mixture (5 ml/5-cm plate) followed by an incubation with 2 ml/pate of this solution. The assay mixture consisted of 70 mM sodium chloride, 30 mM tri(hydroxymethyl)aminomethane, 5 mM magnesium acetate, 0.05 mM ethylenediaminetetra-acetate, 5 mM potassium phosphate, 0.1% (w/v) BSA, and 75 mM glucose (pH 7.2; osmolality: 290 ± 10 mOsm). After 5 min, [γ-32P]ATP was added to 5 μM final concentration, if not otherwise stated. This was rotated over the cells on a rocking platform (15 rpm, Bellco Glass Inc., Vineland) at 37 °C for 10 or 15 min and washed off by 3 ml ice-cold assay mixture without BSA but containing unlabeled ATP (1 mM). This phosphorylation procedure did not alter subsequent growth as followed for 2 days. Rinsed cells were immediately either fixed with 3 ml of ice-cold 5% (v/v) trichloroacetic acid or lysed with sodium dodecyl sulfate-containing solution (see below).

For phosphorylation of exogenous substrates by the intact cells, 1 mg/ml of phosphotitin, histone, or calf serum (10% (v/v) in assay mixture) was added instead of BSA. Controls were performed in the absence of cells.

Phosphorylation of sonicates was performed by incubating cell sonicates (for preparation see above) with [γ-32P]ATP (0.5 μM) at 30 °C for 15 min. The ATP concentration was raised to 5 μM with the unlabeled form and the incubate kept ice-cold until used.

Measurements and Separation Techniques—The radioactivity incorporated in cellular as well as foreign proteins was measured in the trichloroacetic acid-precipitable material or by autoradiography after electrophoresis of the samples in polyacrylamide gels in the presence of SDS.

For trichloroacetic acid-precipitated material, two cycles of resolubilization with ice-cold 1 N NaOH and re-precipitation with 5% trichloroacetic acid were sufficient to remove remaining unbound labeled ATP in agreement with data of Greenaway (11). Radioactivity was determined by counting suitable aliquots in a scintillation counter (Tri-Carb 3380; Packard Instrument Co.). Protein was determined according to the Lowry method (25) with BSA as the standard.

Separation of proteins by electrophoresis in slab gels was carried out essentially according to the method of Laemmli (23). Acrylamide gradients (usually 7.5 to 15%) were overlayered with stacking gel (5% acrylamide concentration). The samples contained final concentrations of 2.5% SDS, 0.05% Tris-HCl (pH 6.0), 30% glycerol, 0.1% dihydroethyriol, a trace of bromphenol blue as tracking dye. The samples were boiled for at least 2 min. After staining and destaining, gels were dried under vacuum and autoradiographed (Cronex 4 x-ray film from Dupont (Frankfurt) or XAR 5 x-ray film from Kodak (Rochester)) or scanned using a thin layer scanner (type II; Berthold, Wildbad). Photometric scans of autoradiograms of gels were performed by a Leitz MPV1 microscope photometer, which was mounted on a modified Berthold thin layer scanner as described previously (19).

Electronic sorting of cells was carried out with a computerized cell sorter FACS II (Beckton Dickinson, Mountain View) equipped with an UV laser. Phosphorylated cultures were removed from the dishes with phosphate-buffered saline solution containing 2 mM EDTA and 1 mM unlabeled ATP (37 °C), the resulting single cell suspensions immersed in phosphate-buffered saline containing 1 mM ATP and the fluorochrome DAPI (3 μg/ml). This compound produces fluorescence predominantly on interaction with double-helical DNA. DAPI enters intact cells considerably slower (in the range of hours) than cells in some way damaged in their membrane structure which are stained immediately. Sorting was carried out on the basis of light-scattering (particle size) and fluorescent intensity. For fluorescent excitation, the laser was tuned to 363 nm at 500 milliwatts; pulses were detected above 390 nm. Sheathing was with 0.15 M NaCl containing 1 mM ATP. The signals were visualized in two dimensions on a storage oscillograph which allowed preselection for different fractions: unstained (intact) cells, stained (damaged) cells, and a fraction in between as shown under “Results.”

Characterization of the Phosphorylated Product—To ascertain the nature of phosphorylated species, trichloroacetic acid-precipitable material was subjected to different agents as described earlier (17). Treatment of trichloroacetic acid-precipitable material with DNase I or RNase A was done as already described (18). In this way, the 32P/14C ratio of cellular material which had been prelabeled by growing the cells in the presence of U-14C-labeled protein hydrolysate (Amersham-Buchler, Hanover) was determined. In other experiments, the sensitivity of phosphorylated products to protease was assayed with 0.05% (w/v) trypsin at 37 °C.

High voltage paper electrophoresis was performed according the method discussed in Ref. 2, as described elsewhere (18) with phosphoserine (Calbiochem, San Diego) and phosphothreonine (ICN, K&K Laboratories, Cleveland, OH) as references. For separation of phosphotyrosine from phosphothreonine, the electrophoresis was carried out at pH 2.8 and pH 3.5 (47) instead of pH 1.9 (acetic acid/pyridine/water, 87:2:9, v/v/v). Unlabeled phosphotyrosine (a gift from Dr. R. Flügel, German Cancer Research Center), phosphoserine, and phosphothreonine were added to the radioactive samples analyzed.

RESULTS

Conditions for and Characterization of the Endogenous Cell Surface Phosphorylation—On incubation of intact HeLa cells (monolayer) under isosmotic conditions in presence of micromolar concentrations of [γ-32P]ATP we observed incorporation of radioactivity into material precipitable by trichloroacetic acid. During the phosphorylation reaction, the capacity of the cells to exclude trypan blue or DAPI and to retain intracellular proteins like lactate dehydrogenase was not altered. Such cultures subsequently continued to grow at their usual rate over a 2-day period.

The amount of precipitable radioactivity generated was dependent on incubation time, cell number, and concentration of [γ-32P]ATP as summarized in Fig. 1. The apparent reaction...
increased with time (Fig. 1a) much as reported earlier for cell surface labeling of a different HeLa line with exogenously added protein kinase (17); comparable kinetics has also been found with mouse fibroblasts (29). The radioactivity incorporated during the linear part of the process, i.e. within the first 10 to 15 min, calculated on a per protein basis ("specific radioactivity") was roughly proportional to the cell number employed. However, if calculated per cell, the radioactivity decreased with increasing cell numbers (Fig. 1b), possibly due to a density-dependent decrease of the protein content per cell (30). With increasing concentrations of ATP (0.125 to 5000 μM) increasing initial velocities were observed. Linear transformation was possible only at low concentrations of ATP (Fig. 1c) which allowed estimation of an apparent $K_m$ value in the order of 2 to 5 μM. The measured phosphoryl group transfer to acid-insoluble material at 24 °C was almost as efficient as at 37 °C, whereas at 12 °C it dropped to about 30%.

Characterization of the Phosphorylated Product—The radioactive trichloroacetic acid-precipitable material was insensitive to DNase and RNase treatment, and resisted extraction with organic solvents (chloroform/methanol, 2:1, v/v), showing that neither nucleic acids nor lipids incorporated radioactivity. The radioactivity was stable in cold alkaline solution (1 N NaOH, 0 °C, 15 min), but 74% was released by hot alkali (1 N NaOH, 100 °C, 15 min) and 41% by hot trichloroacetic acid (10%, 100 °C, 15 min); it resisted hydroxylamine treatment. These data ruled out the presence of acyl phosphate linkages, thus suggesting phosphomonoester linkages (see Ref. 18). Indeed, on partial acidic hydrolysis of the phosphorylated material and high voltage paper electrophoresis, $^{32}$P-serine (main product) and some $^{32}$P-threonine was identified (Fig. 2). P-tyrosine (8, 47) was not detected. Treatment with trypsin (see below) resulted in the generation of $^{32}$P-labeled material of lower molecular weight, consistent with the behavior one would expect from phosphoproteins.

Electrophoretic separation on polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS-PAGE) followed by autoradiography revealed that several proteins carried the $^{32}$P label (Fig. 3). No difference was noted in the phosphoprotein spectrum whether the cells were taken off the dish by SDS, EDTA, or as trichloroacetic acid precipitates. The spectrum did not change significantly on extension of the phosphorylation from 15 to 40 min; the bands, however, became more intensely labeled (Fig. 3, lanes a and b). If a 15-min phosphorylation of HeLa cells was followed by a chase with two washes of prewarmed incubation mixture containing 1 mM unlabeled ATP and further incubation at...

**Fig. 2. Phospho-amino acids from surface-located proteins of intact cells.** Approximately 1.2 × 10⁶ cells were phosphorylated at 37 °C for 20 min. Labeled cells were incubated with trypsin solution (0.05% in 145 mM NaCl) for 5 min at 37 °C. The trypsinate was harvested, centrifuged (3000 × g, 10 min) and the supernatant was precipitated with trichloroacetic acid (10% final concentration). The precipitate was washed with trichloroacetic acid, re-pelleted, hydrolyzed in 6 N HCl for 6 h at 110 °C under vacuum and dried in a rotary evaporator before being subjected to high voltage paper electrophoresis.

**Fig. 3. $^{32}$P-phosphoprotein spectra of intact cells.** Cells were reacted with [γ-$^{32}$P]ATP under different conditions and analyzed on 7.5 to 15% polyacrylamide-SDS gels and by autoradiography. a, b, cells (10⁶/plate) phosphorylated for 15 min and 40 min, respectively; c, cells phosphorylated for 15 min were treated for 4 min with 0.05% trypsin in 145 mM NaCl. d, representative pattern of cellular proteins stained with Coomassie blue which was not detectably altered by any of these treatments. Molecular weight (M₀) of reference proteins: β-galactosidase (135,000); BSA (68,000); ovalbumin (45,000); chymotrypsinogen A (25,000); soybean trypsin inhibitor (20,000); myoglobin (17,800).
37 °C with fresh culture medium containing 0.1% calf serum, neither the specific radioactivity of the cells measured up to 120 min nor the spectrum of phosphoproteins was changed significantly, suggesting a relatively low turnover in vivo.

Evidences for Utilization of $\gamma^{32}$P]ATP at the Cell Surface—Nucleoside triphosphates are usually considered not to penetrate intact biological membranes very easily. Under the conditions employed, micromolar concentrations of extracellular $\gamma^{32}$P]ATP oppose millimolar concentrations of intracellular ATP. Hence, a complete equilibration across the membrane would represent a thousandfold dilution. A thousandfold dilution, however, of extracellular $\gamma^{32}$P]ATP by unlabeled ATP abolished the detection of any radioactive protein. Similarly, the omission of Mg$^{2+}$ from the incubation mixture abolished any detectable protein phosphorylation. $\gamma^{32}$P]ATP arriving inside the cell should find conditions which would allow its utilization even in the absence of extracellular Mg$^{2+}$. But no internal phosphate transfer occurred, although it cannot be excluded that $^{32}$P-labeled ATP may be transported into the cell in the form of MgATP.

The partial hydrolysis of exogenous $\gamma^{32}$P]ATP by intact HeLa cells into $^{32}$P$\gamma$, and $^{32}$P$\beta$, (22) raised the possibility that both the latter might contribute to the detectable phosphorylation by entering the cells and re-forming ATP. Incubation of HeLa cells with comparable amounts of authentic $^{32}$P$\gamma$, or $^{32}$P$\beta$, however, did not yield any phosphorylation pattern.

Localization of Phosphorylated Proteins at the Cell Surface—Treatment of phosphorylated HeLa cells with 0.05% trypsin removed the $^{32}$P label within 4 min (Fig. 3, lane c). Analysis of the trypsinates, i.e. supernatants of cells after trypsin treatment, by SDS-PAGE revealed the generation of small phosphopeptides (Fig. 4).

In such experiments, the intactness of cells is of crucial importance since some doubt has arisen that trypsin removes phosphoproteins only from the surface. We observed that damaged cells in cultures with a high trypan blue index (see below) were also “cleaned” from radioactivity by trypsin to a considerable degree, although in their case, the assumption is that intracellular proteins are also heavily labeled (data not shown). Trypsin-induced lability thus seems to be valid as an indicator for the surface location of proteins only if the intactness of cells has also been proven.

The assumption that the generation of phosphoproteins occurred at the surface of intact cells was further supported by experiments with HeLa cultures differing in their degree of damage. As shown in Table I, the specific radioactivity was considerably higher in cultures with a high percentage of damaged cells, if their inability to exclude trypan blue was taken as a measure of damage. The relationship between damage and specific radioactivity was nonlinear. Nevertheless, in all plots of trypan blue indices versus quantity of protein phosphorylation, a finite value of phosphorylation was obtained on extrapolation to zero trypan blue. Extrapolations never led to the diagram’s origin, as would be the case if intact cells were not phosphorylated by their own surface kinase.

In order to gain more direct evidence, intact cells were separated from damaged cells after phosphorylation by the use of an electronic cell sorter. The results of three independent experiments are shown in Table II. Intact cells always exhibited phosphorylation activity. Phosphorylation in damaged cells was 10 to 20 times higher. The calculations are given on a per cell basis since it was noticed that the protein content of damaged cells varied considerably probably due to the difficulty of creating a “standard damaged cell.” From these data, one can calculate that about 10 to 20% of the phosphorylation measured in cultures with a degree of damage of 1% will stem from damaged cells. Analysis of sorted fractions on SDS-PAGE revealed different phosphoprotein pat-
terns (Fig. 5). The comparison showed that the pattern in separated intact cells closely resembled that obtained in optimal cultures.

In order to evaluate the possibility that some of the phosphorylated proteins may have originated from damaged cells and subsequently become attached to intact cells, sonicates of HeLa cells were phosphorylated with [γ-32P]ATP and a titration carried out with intact cell cultures. As can be seen in Fig. 6, no radioactive protein present in the sonicates was retained by the cells. Those phosphoproteins which do appear in gel lane c do not originate from prelabeled sonicate compounds as indicated by non-correspondence of the pattern. A certain amount of [γ-32P]ATP in the sonicate remains unbound. In the case where the larger amount of sonicate was given, this unbound ATP was just sufficient to allow phosphorylation of surface substrates at a detectable level. These are the bands observed in lane c, which correspond to those seen in Fig. 3.

It could be argued that the surface protein kinase activity was taken up from the medium after release from damaged cells. In this case, one would expect a correspondence of increased surface substrate phosphorylation as a result of increased cell damage. In order to discount this possibility, cultures were incubated prior to phosphorylation with HeLa sonicate in amounts equivalent up to 7% damaged cells in the culture. The phosphorylation of surface substrates in such experiments in no case led to an increase of labeling intensity.

The Surface Protein Kinase—To classify the enzyme activ-

| Type of sorted cells                  | P incorporated (pmol/10⁶ cells) |
|--------------------------------------|---------------------------------|
|                                      | Exp. 1  | Exp. 2  | Exp. 3  |
| Nonfluorescent cells (intact)         | 0.013   | 0.008   | 0.008   |
| Fluorescent cells (damaged)           | 0.14    | 0.126   | 0.163   |

Fig. 5. Profile of 32P-labeled proteins of phosphorylated cells sorted electronically into an intact and a damaged portion. Generation of cultures with a purposely high rate of damaged cells, phosphorylation of cultures for 15 min and subsequent separation into intact and damaged portions by cell sorting was performed as given under "Experimental Procedures." Since the sorting procedure took about 4 h, cell phosphorylation was performed in two batches with 2-h interval in accordance with the stability of the phosphorylation pattern. Left side, image on storage oscillograph as defined by fluorescence intensity and light scattering with the electronically preselected areas of cells to be collected. The other cells were discarded. Sorted wells were fixed in trichloroacetic acid (5% final concentration). Comparable amounts of protein-bound radioactivity were separated by SDS-gel electrophoresis (7.5 to 15% gradient). Shown are the photometrical scans of the autoradiograph. Molecular weight markers as in Fig. 3.

Fig. 6. Incubation of cells with 32P-labeled cell sonicate. Sonicate from cell suspension (10⁶ cells/ml) was prepared and phosphorylated as described under "Experimental Procedures." Aliquots from the radioactive sample were incubated with intact cells (1.3 × 10⁶/dish; 37 °C; 15 min). Supernatant fluid was removed; the cells were first washed extensively with ice-cold assay mixture (containing 1 mM ATP), and then analyzed for retained radioactivity by SDS-electrophoresis (7.5 to 15% gel gradient) followed by autoradiography. Data are from cultures which have been incubated with amounts of 32P-sonicate equivalent to a, 0.35%; b, 0.7%; c, 3.5% damaged cells. The corresponding profiles of 32P-sonicate alone are shown in gel lane d, 5 μl of sonicate; e, 10 μl; f, 50 μl.
ity phosphorylation was carried out in the presence of cAMP and the heat- and acid-stable inhibitor protein specific for cyclic AMP-dependent protein kinases. The results are summarized in Table III. Cyclic AMP (5 μM) did not increase protein phosphorylation. However, the reaction became stimulated in experiments where highly damaged cultures were used, indicating the participation of intracellular cyclic AMP-dependent protein kinase in the detectable reaction (data not shown). In optimal cultures, protein phosphorylation was not shown). In optimal cultures, protein phosphorylation was not dependent protein kinase in the detectable reaction (data not summarized in Table III.

Cyclic AMP (5 μM and the heat- and acid-stable inhibitor specific for cyclic AMP-dependent protein kinase (Exp. 2). The amount of protein kinase inhibitor used was sufficient to inhibit 1.4 × 10^5 units of pure catalytic subunit by 50% (for preparation and definition see Ref. 10).

| Exp. | Bt₂-CAMP | Protein kinase inhibitor | P incorporated pmol/mg* |
|------|----------|-------------------------|------------------------|
| 1    | +        | –                       | 0.79 ± 0.01            |
| 2    | +        | +                       | 0.80 ± 0.05            |
|      | –        | –                       | 0.30 ± 0.04            |
|      |          | –                       | 0.31 ± 0.01            |

* Results of 3 cultures/group, ±S.D.

Influence of cyclic AMP and protein kinase inhibitor on the surface protein kinase activity

Table III

Discussion

Attempts to prove or disprove the occurrence of particular enzyme activities at specific topographical sites such as the inner or outer surfaces of cells encounter many difficulties, especially if the enzymatic activity in question may be expected to be present at more than one site. Since sites cannot be studied in an isolated state and remain intact, other experimental designs have to be utilized. For the study of reactions restricted to the cell surface, advantage can be taken of the membrane barrier of intact cells. In this case, the problem is restricted to the cell surface, advantage can be taken of the membrane barrier in terms of the analytical method employed, but rather whether conditions can be chosen which limit the detectable reaction to the surface. With respect to the surface protein...

Phosphorylation of extracellular phosvitin as catalyzed by cultures with different rates of damage

Total supernatants were removed after 15 min at 37 °C and centrifuged (3000 × g, 10 min). After addition of 2 mg of BSA to the supernatants 4 ml of trichloroacetic acid (10%) was added for precipitation. The precipitates were analyzed for protein-bound radioactivity.

| Cell no./plate | Trypan blue-positive cells | P incorporated pmol/mg* |
|---------------|---------------------------|------------------------|
| 5.0           | 0.1                       | 3.11 ± 0.41            |
| 5.5           | 30.0                      | 2.41 ± 0.95            |

* Results of 3 cultures/group, ±S.D.

Utilization of Exogenous Substrate Proteins—In the presence of ATP (Fig. 7a) or of GTP, HeLa cells phosphorylated externally added phosvitin. Unlike histone (33), phosvitin caused neither a release of intracellular proteins like lactate dehydrogenase nor induction of morphological alterations. HeLa cells could even be grown in the presence of high concentrations of phosvitin (5 mg/ml). The phosphorylation reaction occurred in a time-dependent fashion and was linear up to 10 min. The exogenous substrate competed with cell surface proteins for the ecto-kinase as suggested by an approximately 50% reduction of cell surface phosphorylation in the presence of phosvitin. Phosphorylated phosvitin did not stick to the cell surface as shown in Fig. 7b. If cultures were used with an enlarged percentage of damaged cells, the phosphorylation of phosvitin was diminished (Table IV). This suggests that the phosphorylation of phosvitin is a function of intact HeLa cells only thus further supporting the cell surface location of the enzyme activity.

Another exogenous substrate phosphorylated by the surface enzyme, was a particular constituent of externally added calf serum. This protein had an apparent molecular weight of somewhat above 135,000 (Fig. 7c), and co-migrated with one of the major substrates found after surface phosphorylation of HeLa cells.

**DISCUSSION**

Attempts to prove or disprove the occurrence of particular enzyme activities at specific topographical sites such as the inner or outer surfaces of cells encounter many difficulties, especially if the enzymatic activity in question may be expected to be present at more than one site. Since sites cannot be studied in an isolated state and remain intact, other experimental designs have to be utilized. For the study of reactions restricted to the cell surface, advantage can be taken of the membrane barrier of intact cells. In this case, the problem is not whether the plasma membrane represents an absolute barrier in terms of the analytical method employed, but rather whether conditions can be chosen which limit the detectable reaction to the surface. With respect to the surface protein...
kinase discussed here, the detectable reaction is in fact restricted to the surface, as demonstrated in several ways. A major portion of the demonstration is concerned with the specific movements of $[\gamma-^32P]ATP$. The concentration of $[\gamma-^32P]ATP$ in the medium is in the micromolar range; intracellular ATP concentrations are at the millimolar level. This means any labeled ATP penetrating the cell is instantly diluted if it is assumed internal ATP lacks compartmentalization. Experiments in which phosphorylation was carried out with a thousandfold excess of cold over labeled ATP in the external medium indicate that such a dilution effectively prevents measurable transfer of $^32P$. On the other hand, even if sufficient quantities of $[\gamma-^32P]ATP$ could somehow enter intact cells, one would expect subsequent internal generation of labeled proteins to be independent of the presence of Mg$^{2+}$ in the extracellular medium. But extracellular Mg$^{2+}$ at the millimolar level was an absolute requirement for a detectable protein kinase reaction. Finally, it could be argued that extracellularly generated $^32P$, or ATP (22) was taken up by the cells and was the source for intracellular protein phosphorylation. However, 5 mM unlabeled phosphate included in the reaction medium prevented detectable $^32P$ utilization.

Conclusive evidence that $[\gamma-^32P]ATP$ was utilized at the cell surface was obtained by evaluating the degree of interference caused by cells in which the membrane barrier was not intact, a condition also indicated by the uptake of trypan blue. These damaged cells have lost much of their intracellular ATP and cannot appreciably dilute the isotope; in these cells intracellular protein kinase and substrates are accessible to radioactive ATP. It can be calculated from the data shown that in optimal cultures (0.5 to 1% trypan blue-positive cells) over 80% of the phosphorylated material belongs to intact cells and less than 20% stems from damaged cells. These values could also be obtained by direct measurement after electronic cell sorting, which in addition revealed qualitative differences in the spectrum of labeled proteins between damaged and intact cells. Once this proportion is known, it was possible to interpret experiments using labeled material in the presence of trypsin as an indicator of cell surface location, correcting for the tendency of the trypsin to remove intracellular phosphoproteins from the damaged cells. It was found that in no case could participation by enzyme or substrates from the damaged cells account for the level of phosphorylation observed. Further, the results obtained with cell sonicates mimicking different levels of culture damage do not provide the least evidence for a transfer of phosphorylated proteins, substrates, or enzyme activity from damaged cells to the surface of the intact cells. Such data make it certain that ecto-protein kinase exists at the HeLa cell surface and mediates the phosphorylation of cell surface proteins.

The enzymatic character of the phosphorylation reaction was indicated by its dependence on cell number, temperature, and ATP concentration. The $K_m$ for the co-substrate in the order of 2 to 5 $\mu$M is in the range of that found for a similar enzyme activity isolated from HeLa cells (32). For the experimental design this would mean that about $10 \times K_m$ concentrations were necessary in order to approach "saturating" conditions (9). This was not possible, however, since ATP in such concentrations exerts pharmacological effects on cells in culture (1, 5, 36, 37, 43). In order to exclude such "side reactions," we continued with ATP concentrations in the range of $1/10 \times K_m$.

The products of enzymatic activity were phosphoproteins bearing the phosphoryl groups esterified to serine and threonine residues but not to tyrosine residues as catalyzed by the src gene protein kinase (8, 14, 47). Radioactively phosphorylated nucleic acids or lipids were not detected, nor were acyl phosphate linkages characteristic for intermediates of ATPase reaction (31) or acid-soluble phosphoamidates (41).

The ability of GTP to act as co-substrate, and the ineffectiveness of cAMP and of the heat- and acid-stable inhibitor specific for the catalytic subunit of cAMP-dependent protein kinase (44) placed the enzyme outside the group of cyclic nucleotide-dependent protein kinase. Phosphorylation of the exogenous substrate phosphoinositide by intact cells characterized the enzyme further and indicated its surface location. Collectively, these data identify the enzyme as a non-cyclic nucleotide-dependent protein kinase.

Unlike phosphoinositide, histones could not be examined for their ability to serve as substrates for the surface protein kinase since even low concentrations of histones have been shown to affect drastically the intactness of membrane barrier as indicated by release of the cytosolic enzyme lactate dehydrogenase and cAMP-dependent protein kinase activity. Clumping of Ehrlich ascites cells (35), cytology of 3T3 (28), or Dictyostelium (33) caused by histones indicate that this may be a general response. The use of histones, therefore, cannot be recommended, as it violates the requirement for an intact membrane barrier, although in a variety of studies (16, 26, 34, 39) which appeared during the progress of this work, histones have been used as substrates for postulated ecto-protein kinase activity.

The physiological role of ecto-protein kinase activity remains to be established. It could have two possible functions: the modulation of the cell surface of the same cell or, presumably especially important for cells of multicellular organisms, interaction with the microenvironment, extracellular fluids, neighboring cells or structures. Unlike proteases, protein kinases alter proteins in a reversible manner. The demonstration of ecto-phosphoprotein phosphatase activity in 3T3 cells (28), although not in HeLa cells as yet, may point to a regulatory system as inherent in phosphorylation-dephosphorylation mechanisms described for a variety of proteins. With regard to this, it is interesting that when calf serum was added to the medium only a single protein was heavily phosphorylated. This is a remarkably specific response when compared to the wide spectrum of serum proteins phosphorylated by cAMP-dependent protein kinase (19). This protein had an apparent molecular weight of ~135,000. It was also detected among the cell surface proteins but was readily released into the extracellular fluid.

Chase experiments with the labeled products of ecto-protein kinase revealed that they were not easily dephosphorylated which was also indicated by the stability of the phosphoprotein spectrum. This result is surprising in view of the fact that other membrane-bound phosphoproteins show a relatively high turnover of the phosphoryl groups (4). This suggests that the ecto-protein kinase might be involved in steady state processes rather than in events which are regulated by fast phosphorylation.

Hints as to the functions of an ecto-protein kinase may be given by effects in intact cells of small amounts of extracellular ATP (1, 5, 6, 21, 36, 37, 42, 43). Extracellular ATP has been shown to modify transport properties of cells (5, 27, 36, 37). In view of a recently reported pyrophosphate-protein phosphotransferase bound to membranes (24) and our own observation that pyrophosphate can be generated at the surface of HeLa cells (22), it remains to be established whether the ecto-protein kinase activity reported here consists of one or more entities.

Work is in progress to characterize the ecto-enzyme activity

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of cells as well as the main surface-located substrate proteins in order to gain direct insight into the functions of this system.

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