Several types of cells exhibit cell surface protein kinase (ecto-PK) activities with Ser/Thr-specificity. Ecto-PK sharing certain characteristics of protein kinase CK2 can be detached from intact cells by interaction with exogenous substrates (Kübler, D., Pyerin, W., Burow, E., and Kinzel, V. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 4021–4025). However, a detailed molecular analysis of this ecto-PK was hampered by the vanishingly small amounts of labile enzyme protein obtained by substrate-induced enzyme release. We now describe the stabilization and enrichment of released ecto-PK by precipitation with polyethylene glycol followed by affinity chromatography on heparin-agarose. Ecto-PK is shown to consist of two separate forms released in tandem, ecto-PK I and ecto-PK II. Comparison with cell homogenates as well as cell surface biotinylation experiments excluded contamination with intracellular PK. Purified ecto-PK I and ecto-PK II exhibit respectively selective phosphorylation of CK1- and CK2-specific peptide substrates, a complementary sensitivity to inhibitory agents and a differential use of the cosubstrates ATP and GTP. Ecto-PK I consists of a 40-kDa moiety; the ecto-PK II is an ensemble of three components of 43- and 40-kDa (catalytic subunits) and a noncatalytic 28-kDa subunit. In addition, components of the ecto-PK II react with CK2-specific antibodies. Further, comparative peptide mapping and the results of mass spectrometry in combination with assignment of amino acid sequences confirmed that ecto-PK II is closely related if not identical to the protein kinase CK2. Assays with intact cells that result in the phosphorylation of a variety of endogenous membrane proteins showed that both ecto-PKs participate, and further, certain ecto-PK substrates become preferentially labeled by one or another of the enzymes, whereas others are phosphorylated by both ecto-PK activities.

The activity of protein kinases (PK) is well established as a major mediator by which cells relay important signals for cell growth, metabolism, and homeostasis. The recognition of its potential importance for extracellular events, however, is relatively recent. Cell surface PK (ecto-PK), due to their exposed location, have a potential for reception and transduction of external stimuli. Using extracellular ATP, the ecto-PK activities allow phosphorylation of cell surface proteins and/or soluble external substrate proteins in the environment of the cells. Ecto-PK of eucaryotic cells has been related to a number of biological phenomena (1–8); also, certain parasitic protozoa seem to use ectophosphorylation for interaction with host cells (9). The biological relevance of ecto-PK and extracellular protein phosphorylation received complementary support from abundant evidence for the existence and the biological activity of the cosubstrate ATP external to cells, partly transduced by specific surface receptors (for a recent review, see Ref. 10).

Studies in our laboratory and those of others have shown cyclic nucleotide-independent and cAMP-dependent types of ecto-PK activities in a wide range of vertebrate cell types (11–15). A ubiquitous ecto-PK activity, insensitive to cyclic nucleotides or Ca²⁺, enables viable cells to phosphorylate endogenous membrane proteins and foreign substrates. The enzymes’ properties agree with those known for intracellular protein kinase CK2 insofar as acidic prototype substrates were phosphorylated with ATP and GTP as the phosphoryl group donor and the enzymes displayed sensitivity to the glycansulfoglycan heparin (11, 16). A unique feature is that this ecto-PK can be released from the intact cell through exogenous protein substrate (17). The inducible discharge of cell surface PK is dependent on stimulation by exogenous substrate and occurs instantly, thus differing basically from exocytosis or spontaneous shedding. A series of specific criteria established by our early studies have shown that ecto-PK shedding occurs in a selective manner, including no intracellular components (11, 12, 17–20).

Structural as well as functional characterization of ecto-PK activities and their appropriate substrates are only just beginning. Recently, we succeeded in the isolation and identification of two major ecto-PK substrates on the cell surface, revealing them to be homologous forms of certain nuclear proteins (21). On the other hand, the only direct approach to isolation of ecto-PK is the technique of substrate-induced release, which yields at best vanishingly small amounts of enzyme protein. Hence investigations of the molecular properties of the ecto-PK itself are difficult unless sufficient amounts of enzyme protein are available.

The present study aimed at the characterization of substrate-detached ecto-PK from intact HeLa cells was made possible by the development of a concentration procedure for a simultaneous storage and accumulation of enzyme protein for this purpose. Comparison of the data with known intracellular PKs established that two related ecto-PK forms exist at the cell surface and were set free in tandem. Knowledge of their characteristics will be advantageous for the future detection of specific ecto-PK substrates and the role of their phosphorylation.
EXPERIMENTAL PROCEDURES

Materials—Peptides specific for protein kinase CK1, (DDDD-VASLPGRLR) (22), RKKDLDHDEEAMISITA) (23), and protein kinase CK2, (RRRAADSDDDDD (24) and RRREREEEETEEEEE (23)), were prepared by the peptide synthesis unit of the DKFZ. Ethidium bromide and the material for enhanced chemiluminescence (Western-Light-Detection kit) was obtained from Serva (Heidelberg, FRG). All other reagents and chemicals were of the highest grade available. Protein kinase CK1 isolated from rat liver was kindly supplied by the group of Prof. O. Issinger (University of Homburg, FRG).

Cell Culture—Cells of Hela monolayer cells were adapted to and finally cultivated in serum-free HL-1 medium (Ventrex). Briefly, adaption was carried out by a stepwise reduction of calf serum (5, 2.5, 1, or 0.5% or no serum) with two passages at each step. Serum-free grown cells have been kept for an as yet unlimited number of passages (90). For experiments the cells were plated in 25- or 175-cm² tissue culture flasks (Falcon) and raised to densities of 4–6 × 10⁶ cells/cm² (subconfluent cultures). Cell viability was evaluated after exposure of the cells to conditions for the substrate-inducible release of ecto-PK activity by a number of independent criteria as described earlier (17).

Cell Sonicates—Cell sonicates (representing intracellular PK activities, used for experiments as detailed in Figs. 1 and 7) were obtained by scraping off the cells (10⁶ cells/ml of chilled buffer) from the culture flask in the identical buffer used for ecto-PK release (see below) including 0.5 mg/ml phosphatase. The cell suspension was sonicated (Branson cell disruptor B15; microtip, step 3; 7-s blasts with 10-s intervals) for 2 min and subsequently passed through a 0.2-μm sterile filter (Millipore) to clear it of particulate material; the filtrate referred to as cell sonicate was kept ice-cold until use.

Release of Ecto-PK Activity from Intact Cells into the Supernatant—Release of ecto-PK activity from intact cells into the supernatant was carried out in the presence of 0.5 mg/ml phosphatase for 10 min at 37°C as described previously (17). The supernatant was passed through a 0.2-μm sterile filter (Sartorius) to remove detached cells or any other particulate matter. The cleared supernatants were treated further for enrichment and purification as given below. Small aliquots of this supernatant served to determine PK activity.

Preticipation of Ecto-PK by Polyethylene Glycol—Cell-free ecto-PK preparation was mixed with polyethylene glycol (PEG 6000) at concentrations indicated in the individual experiment. The solutions were kept on ice for 45 min before the precipitates were collected by centrifugation (12000 × g for 10 min). Supernatants were discarded, and the pelleted material was stored at −80°C. PK assays (phosphatase phosphorylation) with material redissolved in P-Mix (see above) showed that PK activities could be fully preserved as PEG precipitate.

Heparin Affinity Chromatography—Heparin affinity chromatography was carried out with heparin-agarose (column size, 1 ml; Pharmacia) using the technique of fast protein liquid chromatography (Pharmacia). The Column was equilibrated with buffer (50 mM NaCl, pH 7.4, 10 mM sodium acetate, 2 mM EDTA, 1.5 mM 1,4-dithiothreitol; and 0.2 mM phenylmethylsulfonyl fluoride), and samples were loaded (0.7 ml/min) on a column that had been pre-equilibrated with buffer at 100 mM NaCl. The column was extensively washed with the same buffer until the effluent was essentially free of protein. Elution was performed with buffer (as above) containing a linear gradient of 0.1–1 N NaCl. Fractions of 2 ml were collected and, after adjusting the samples to 0.1 N NaCl by dilution, assayed for PK activity as given below. Fractions with the highest kinase activities (see Fig. 2) were used for further characterization. If necessary, the eluate fractions were concentrated in batches of 2 ml by ultrafiltration (Centricon-10, Amicon).

Phosphorylation Assays—Phosphorylation of phosphotyrosine by released ecto-PK and analysis of incorporated radioactivity by liquid scintillation counting were as described earlier (12). Optionanly, the phosphorylation reaction was carried out in the presence of either the protein kinase CK1 inhibitor CK1 -1 (Sagakgau) or the CK2 inhibitor heparin (Riker-Ketehak) at concentrations given in the particular experiments.

Autophosphorylation of Ecto-PK—Enzyme-specific autophosphorylation was measured with Ponzoine S or Amido Black.

Protein Phosphatase Assays—Phosphorylation of phosphotyrosine by released ecto-PK and analysis of incorporated radioactivity by liquid scintillation counting were as described earlier (12). Optionanly, the phosphorylation reaction was carried out in the presence of either the protein kinase CK1 inhibitor CK1 -1 (Sagakgau) or the CK2 inhibitor heparin (Riker-Ketehak) at concentrations given in the particular experiments.

Autophosphorylation of Ecto-PK—Enzyme-specific autophosphorylation was measured with Ponzoine S or Amido Black.

Western Immunoblot Analysis—Proteins separated by SDS-PAGE were electroblotted onto a nitrocellulose membrane using the semi-dry system described by Kysehra-Anderson (29). Protein on PVDF membranes was stained with Coomassie blue. A colored, rehydrated, dried gel was exposed to a x-ray film for detection after autoradiography.

For immunodetection, the PVDF membranes were incubated with specific polyclonal antibodies. Primary antibodies were stained by enhanced chemiluminescence (Western Light Detection kit) using alkaline phosphatase-conjugated secondary antibodies and its specific substrate bisodium 3-[4-methoxybenzilidene]oxazolin-2(3'-H) chromo (3, 1, 1-decan)4-phenylphosphate. Signals were detected by exposure to x-ray film.

Peptide Mapping by Tryptsin Digestion—Peptide mapping by trypsin digestion of proteins blotted to PVDF membranes were carried using the semi-dry system described by Kysehra-Anderson (29). Briefly, membranes were cut into small pieces (1 × 1 mm) and incubated with 100 μg Tris/HCl, pH 8/0.1% acetonitrile. The membranes were transferred to an RTX-100, including 0.1 mg of trypsin (Boehringer Mannheim, sequencing grade) of protein for 24 h at 30°C. Tryptic peptides were desorbed from PVDF membranes with 0.1% trifluoroacetic acid under sonification, and collected supernatants were stored at −20°C.

HPLC Chromatography—Tryptic peptides were loaded onto a reverse phase HPLC column (C18, Aquapore OD-300 GLB-22; 22 × 0.21 cm; 7 mm; Applied Biosystems), washed with 0.1% trifluoroacetic acid, and eluted (100 ml/min at 70 bar) with a linear gradient of 0–80% acetonitrile/0.08% trifluoroacetic acid. Peptides were monitored at 220 nm.

N-Terminal Microsequencing—Proteins were separated by SDS-PAGE and transferred to PVDF membranes. After incubation by staining with Ponzoine S, the desired proteins were cut out and after drying stored at −20°C until use. Blotted proteins were applied to an automated 477A protein sequencer (Applied Biosystems) and assayed for...
Preparation was separated by a 0.1–1 M NaCl gradient into two fractions. As shown in Fig. 1, the ecto-PK was separated from intact cells (2 × 10^9) without or with 0.5 mg/ml phosvitin and precipitated with 10% PEG as described under "Experimental Procedures." The precipitates were resolved in chromatography buffer including 0.1 M NaCl, and samples were loaded to a heparin-agarose column (see "Experimental Procedures"). After extensive washing with the same buffer, the column was eluted with a linear gradient of NaCl (0.1–1 M), and fractions of 2 ml were collected. Aliquots of each fraction were assayed for PK activity with [γ-^32P]ATP or [γ-^32P]GTP and phosvitin as the substrate as described under "Experimental Procedures." Shown are PK activity profiles from cell supernatants obtained in the presence (●) or the absence (○) of phosvitin. The inset shows the profile obtained from cell sonicates (obtained from 2 × 10^9 cells) that represent intracellular PK activities. It should be noted that the activity levels here are significantly higher.

**RESULTS**

Purification of Ecto-PK—Intact HeLa cells grown under serum-free conditions were incubated with phosvitin (0.5 mg/ml) for release of ecto-PK activity ("substrate inducible shedding"). Substrate-induced release yielded less than 20 ng of ecto-PK enzyme protein/10^6 cells as estimated on the basis of a purified CK2 preparation of known specific activity. In addition, ecto-PK solutions proved to be rather unstable, because the phosvitin phosphorylating activity disappeared within a few hours (data not shown). To enrich enzyme ecto-PK and preserve its enzymatic activity, enzyme protein was precipitated with PEG. The kinase activity could be quantitatively precipitated together with phosvitin by PEG at concentrations above 10% and subsequently recovered with no loss of enzyme activity. PEG-precipitated ecto-PK, even after storage at −80 °C for up to 1 year, could be restored to full activity.

To remove the large surplus of phosvitin, heparin-agarose was used. It has a high affinity for ecto-PK whereas the bulk protein, phosvitin, does not bind to this matrix to any significant extent. Material from a routine ecto-PK preparation (10^8 cells) after resolubilization of PEG precipitate was subjected to heparin-agarose column chromatography as described under "Experimental Procedures." As shown in Fig. 1, the ecto-PK preparation was separated by a 0.1–1 M NaCl gradient into two PK activity peaks when assayed with [γ-^32P]ATP. The first peak of phosvitin phosphorylating activity (peak I) eluted at about 0.6 M NaCl; the second activity (peak II) eluted slightly above 0.8 M NaCl. When the phosvitin phosphorylation was conducted with [γ-^32P]GTP instead of [γ-^32P]ATP, it was observed that the peak II fractions utilized this cosubstrate, corresponding to the known capability of CK2 to use GTP. Ecto-PK I underwent an approximately 9000-fold enrichment with an approximately 22000-fold purification for ecto-PK II (Table I). Separation of the ecto-PK fractions by SDS-PAGE revealed that both fractions carried several proteins.

Equivalent amounts of cell homogenate (rather than material from intact cells) complemented with phosphitin under identical conditions for PEG precipitation and heparin affinity chromatography (see "Experimental Procedures") and exhibited kinase activity profiles with three phosphitin kinase activity peaks (Fig. 1, inset). Besides the activities corresponding to peak I and peak II, another major peak of phosphitin phosphorylating activity was eluted ahead of peak I at −0.5 M NaCl. It is important to note that about 200-fold higher levels of enzyme activity were obtained with homogenates from a given number of cells than with supernatant material from the same number of intact cells. Hence the difference observed between enzyme activity profiles under both conditions eliminate the possibility of participation by material from damaged cells to the activity profile of released ecto-PK. This was confirmed by using cell surface biotinylation for further control of cell surface origin.

Enzymatic Properties of Affinity Purified Ecto-PK—Further characterization of the affinity purified ecto-PK activities was carried out with particular peptide substrates specific for CK1 (22, 23) and CK2 (23, 24). The CK1 peptide but not the CK2 peptide was phosphorylated by the ecto-PK I activity (Fig. 2, lane 1). Ecto-PK II exclusively phosphorylated the CK2-specific peptide (Fig. 2, lane 2). The same trend was obtained when using other specific peptide substrates, the CK1 peptide RRK-DLHDDEEEAAAMSTA and the CK2 peptide RRREEETEEE. This result correlates ecto-PK I activity with CK1 and ecto-PK II activity with CK2, respectively. Control experiments with authentic intracellular CK1 (from rat) and CK2 (human) confirmed these relationships (Fig. 2, lanes 3 and 4).

The evaluation of the ecto-PK properties was extended using inhibitors of casein kinases, including the isoquinolin derivative CK I-7 for CK1 and heparin for CK2. As shown in Fig. 3, CK I-7 preferentially inhibits ecto-PK I activity, and heparin affected ecto-PK II catalyzed phosphorylation. The 50% inhibition (I_{50}) of ecto-PK I peptide phosphorylation was obtained at 0.075 μM CK I-7, whereas this inhibition was not reached in the ecto-PK II assay. Conversely, the I_{50} values for heparin were determined as 0.16 μg/ml for the ecto-PK II phosphorylation and were indeterminable in the ecto-PK I assay. These results confirm the identity of ecto-PK.

**Molecular Properties**—Autophosphorylation in solution (see "Experimental Procedures") with the ecto-PK I preparation revealed labeling of a 40-kDa polypeptide. However, in the presence of polylysine, a modulator of protein kinase CK2 activity (32), the labeling of the 40-kDa component was suppressed, whereas two other polypeptides of 20 and 21 kDa became phosphorylated instead (data not shown). Causally this result indicates the presence either of enzyme subunits or of other substrates in the enzyme preparation. In the case of ecto-PK II activity, three proteins of 43, 40, and 28 kDa were radiolabeled, but phosphorylation of these proteins was abolished in the presence of polylysine (not shown). Because of the presence of more than a single phosphorylated polypeptide in the ecto-PK preparations, it was imperative to determine which protein represented the catalytic portion of the enzyme.
Ecto-Protein Kinases CK1 and CK2

Summary of ecto-PK purification through heparin-agarose chromatography

Table I

| Volume | Protein | Total activity | Specific activity | Purification factora |
|--------|---------|----------------|-------------------|---------------------|
| ml     | mg      | units          | units/mg          | fold                |
| Starting material | | | | | |
| ecto-PK I | 45 | 180b | 1232 | 6.8 | 1 |
| ecto-PK II | 6 | 0.004c | 247 | 24700 | 9080 |

a Calculation was based on the proportion of total activities of ecto-PK I and ecto-PK II after their separation on heparin-agarose (as in third column).

b These values mainly represent phosphoribosyltransferase that has been added for the purpose of ecto-PK release.

c Protein measurement was done from comparative protein staining on SDS-polyacrylamide gels (Coomassie) or after western blotting to PVDF membranes (Amido Black) with authentic CK2 as the reference.

FIG. 2. Comparative phosphorylation of specific peptide substrates by purified ecto-PK and intracellular PK activities. Phosphorylation assays using the peptide substrates (indicated by single letter code) were carried out with 1 μM [γ-32P]ATP for 12 min as described under “Experimental Procedures.” All inputs (2 μl) of the radioactive reaction mixture were analyzed by thin layer chromatography on cellulose plates and high voltage electrophoresis. Radioactivity was detected by autoradiography. Shown are 32P-labeled peptides phosphorylated by affinity purified ecto-PK I (lanes 1 and 2) and ecto-PK II (lanes 2). Peptide phosphorylation by authentic protein kinases CK1 (lanes 3) and CK2 (lanes 4) served as reference. Positions of radioactively labeled peptides and free [γ-32P]ATP are indicated.

FIG. 3. Effects of CK I-7 (A) and heparin (B) on the activity of ecto-PK. The conditions of the phosphorylation reactions with the specific peptides DDDDVASLPGLRRR by ecto-PK I (●) and RRAADSDDDDD by ecto-PK II (○) and the measurement of peptide phosphorylation by cellulose thin layer electrophoresis were performed as described in the legend of Fig. 2. Phosphorylation rates were measured in the presence of CK I-7 or heparin at concentrations given in the graph. The mean values of four independent experiments are given ± S.D.

To address this problem we took advantage of a PK renaturation test on substrate-containing SDS-polyacrylamide gels (in gel assays) as described under “Experimental Procedures.” The results show that ecto-PK I fractions (Fig. 4A) in the presence of [γ-32P]ATP label a single phosphoprotein of 40 kDa (lane 1), indicating that this band represents the ecto-PK I activity. In the case of ecto-PK II, the 43- and 40-kDa components were labeled (lane 2). In contrast, the 28-kDa polypeptide detected by autophosphorylation assay in solution (above) was not labeled under these conditions, suggesting either the noncatalytic subunit or copurified substrate. When the in gel assay was conducted with [γ-32P]GTP, the ecto-PK I sample did not autophosphorylate (Fig. 4B, lane 1), as expected for CK1 enzymes. In contrast, the ecto-PK II 43-kDa component as well as the 40-kDa polypeptide (lane 2) could use GTP and became autophosphorylated.

Further Western immunoblot analysis was carried out with a collection of CK2 antibodies against the subunit α, α', and β (Fig. 5). None of the CK2 antibodies recognized ecto-PK I (lane 1). However, ecto-PK II produced positive signals (lanes 2-5) that, in agreement with the control CK2 holoenzyme (lane 6), showed the 43-, 40-, and 28-kDa proteins to be α, α', and β subunits. The determination of the heteromeric composition of ecto-PK II remains to be elucidated. On the other hand, that ecto-PK I proteins failed to be stained by any of the CK2 antibodies further indicates the separate nature of the ecto-enzymes under investigation (no antibodies toward human forms of CK1 are available at this time).

Because N-terminal amino acid sequences from affinity purified ecto-PK I and II blotted to PVDF membranes (Amido Black) with authentic CK2 as the reference.


these enzymes, a valid identification on this basis can only be made with reference to a known sample also of human origin; when human CK2 is available, human CK1 is not.

For determining internal peptide sequences, ~30 pmol of the 43-kDa subunit of ecto-CK II was digested with trypsin. Proteolytic peptides were separated by reversed-phase HPLC on a C18-column (see "Experimental Procedures"). For comparison, recombinant human protein kinase CK2α subunit was prepared and digested. A total of 17 trypsin peptides, referred to as #1 to #17, were resolved by the HPLC. Fig. 6 shows very similar HPLC peptide profiles of the α subunit of ecto-CK II (A) versus control CK2α (B), suggesting a highly homologous if not identical composition of the two enzyme forms. The peptides #9–11 and #14–17 of both the ecto-CK and the CK2α were chosen for further analysis by mass spectrometry (MALDI-MS; see "Experimental Procedures"). The molecular masses of the tryptic peptides were found to be very similar. In addition, the tryptic peptides #9, 14, and 15–17 could be matched by computer-assisted analysis with theoretical partial amino acid sequences derived from human CK2α (Table I). Using this combination of MALDI-MS and sequence determination, at least 27% of the total amino acid sequence of catalytic subunit of the ecto-CK II was identified.

Cell Surface Biotinylation Separates Ecto-CK I and Ecto-CK II from Their Related Intracellular Enzymes—That ecto-CK is released from the surface of intact cells and not derived from intracellular sources was retested by using the method of cell surface labeling with biotin (see "Experimental Procedures"). Biotinylated cells were treated under routine conditions for ecto-CK release with phosvitin, and cell supernatants were harvested. Subsequently the cells after ecto-CK release were treated by sonification (in the presence of phosvitin) to obtain intracellular kinases. Both types of samples, supernatants from intact cells and the cell sonicates, were then treated in parallel by PEG precipitation and heparin affinity chromatography. The results (Fig. 7) clearly showed that the ecto-CK I (A, lane 2) was biotinylated, whereas the corresponding material from sonicated cells was not (A, lane 1). Similarly, ecto-CK IIα was biotinylated but not the material of the cell sonicate (B, lanes 1 and 2). These results, together with the previous ones, form compelling evidence for the cell surface origin of the released ecto-CKs.

Cell Surface Protein Substrates of Ecto-CK I and Ecto-CK II—The identification of two separate ecto-CK activities prompted us to study their substrate specificity further, particularly toward the panel of endogenous membrane proteins that become phosphorylated after incubation of intact cells with extracellular [γ-32P]ATP (see the Introduction). Because the specific inhibitors of CK activities, CK I-7 and heparin, differentially affect the cell-free forms of ecto-CKs (see Fig. 3) these agents could provide a potential means of partitioning cell surface protein phosphorylation on intact cells as well. To investigate this possibility, intact cells were surface phosphorylated under the influence of 50 μM CK I-7 and 3 μg of heparin, under which condition substrate phosphorylation was found to be significantly reduced (as in Fig. 3 above). Fig. 8
Ecto-Protein Kinases CK1 and CK2

tryptic peptides were obtained as described under "Experimental Procedures" and in the text. Masses of the peptides were determined by using the MALDI-MS system described under "Experimental Procedures." Identification of the peptides was done by computer-assisted analysis using the SWISSPROT sequence data bank and the program package HUSAR (see "Experimental Procedures"). The calculated monoisotopic masses (MH) of tryptic products of human CK2α were used as the reference.

| Peptide number | Observed MH of ecto-PKII | CK2α |
|----------------|--------------------------|------|
|                | Observed MH               | Calculated MH |
| 9              | 1527.1                    | 1527.7          |
| 10             | 1730.9                    | 1731.8          |
| 11             | 964.9                     | 965.2           |
| 14             | 1009.0                    | 1008.8          |
| 15             | 2830.6                    | 2830.4          |
| 16             | 2323.6                    | 2324.1          |
| 17             | 2305.4                    | 2303.9          |

**Fig. 7. Labeling of ecto-PK by cell surface biotinylation.** HeLa cultures (6.4 x 10⁶ cells total) were surface-labeled with N-hydroxysuccinimidyl-biotin under the conditions described under "Experimental Procedures," and ecto-PK was released from biotinylated cells with phosvitin under the routine conditions described in the legend of Fig. 1. To obtain intracellular PKs, the cells after ecto-PK release were washed twice with isotonic buffer, scraped from the bottoms of culture flasks, and disrupted by sonication (see "Experimental Procedures"). Released ecto-PK from cell supernatants and intracellular PKs from cell sonicates were proceeded through heparin-agarose chromatography followed by SDS-PAGE and transfer to PVDF membrane. Biotinylation was detected by the enhanced chemiluminescence technique with alkaline phosphatase-conjugated streptavidin (see "Experimental Procedures"). The labeling of material separated by heparin-agarose is shown for peak 1 activities (A) and peak II activities (B). Lanes 1 show intracellular PKs from cell sonicates; lanes 2 show the ecto-PK isolated from intact cells. The relevant part corresponding to the location of the catalytic subunits of 40 and 43 kDa is presented.

Table II

| Peptide number | Amino acid sequence (position in CK2α) |
|----------------|----------------------------------------|
| 9              | QLYQTLTDDYDIR (123–134)                 |
| 10             | TPALVEHVNNTDFK (108–122)               |
| 11             | EYWDYESHVVEQGDYQLVR (22–43)            |
| 14             | FHVSENQHLVPSAEFDLDK (284–303)          |
| 15             | LIDNGLAEFVIPQGYNNV (173–191)           |
| 17             | VLGEDLYVDYKYNIELDPR (248–268)          |

* i.d., indeterminable.

**DISCUSSION**

The release of ecto-PK from intact cells by protein kinase substrates such as phosvitin or casein appears to be a common phenomenon (17, 33, 34). The ecto-PK shedding occurs as a specific and immediate response of intact cells to stimulus by a protein substrate. At present, the mode of membrane anchoring of the ecto-PK or the mechanism underlying the enzyme release are not known. However, previous experiments (33, 35) have ruled out the possibility that phosphatidyl inositol-specific phospholipase C could cleave ecto-PK activity from intact cells, which excludes a glycosyl phosphatidylinositol anchor such as described for some other cell surface-located proteins (36). An ecto-PK liberation by specific proteolysis is unlikely because several protease inhibitors with different specificities were not able to suppress enzyme release (35, 37).

The present study adds important criteria that support the evidence for the cell surface origin of the ecto-PK and the specificity of the substrate-dependent ecto-PK shedding and discount the possibility of a contribution by intracellular PK activities from dead or damaged cells (11, 12, 17). Firstly, the experiments here were carried out with HeLa cells grown in serum-free medium to reduce any unspecific protein load of the cell supernatants, because serum protein components may stick firmly to cell cultures. Secondly, comparative affinity chromatography with cell supernatants from intact cells and material from cell homogenates treated under identical conditions resulted in different activity profiles having significantly different activity levels. Thirdly, specific cell surface biotinylation resulted in the labeling of both ecto-PK forms, although their corresponding intracellular PK stayed unlabelled.

In the case of ecto-PK I, a relation to protein kinase CK1 was brought out directly by phosphorylation assays and indirectly by the absence of properties exhibited by the second ecto-PK released from intact cells, ecto-PK II. Confirmation of the classification was obtained by specific phosphorylation of the CK1 peptide substrates, DDDDVASLPGLRRR and RRKDLHDDEEDEAMSITA, and through sensitivity to CK I-7, a specific CK1 inhibitor. That the ecto-PK I-catalyzed phosphorylation reactions were limited to the use of ATP as the cosubstrate agrees with the other properties common for CK1 enzymes and is also in line with authentic CK1 from rat, which served as the control CK1 enzyme in this study.

Protein kinases CK1 have been described as an ubiquitous enzyme family implicated in the control of cytoplasmic and nuclear processes (38–41). Molecular analysis has shown the existence of related yet distinct mammalian CK1 isoenzymes, α, β, γ, and δ in rat brain and testis (ranging in size from 25 to 55 kDa), which most probably represent separate gene products (42–44). Although certain isoforms appear to have broad substrate specificity, the possibility of a different subcellular distribution of these enzymes is not well studied. CK1 forms in yeast carry a prenylation motif (XCC) at their C terminus (45) that might aid their location at the plasma membrane (46).

Recently two members of the human CK1 gene family were described (47, 48). Whether the ecto-PK I (ecto-CK1) represents these or one of the other CK1 family members will require additional characterization at the molecular level.
The identification of ecto-PK II as a protein kinase CK2-like enzyme was proven by the specific phosphorylation of the CK2 peptides RRREEETEEE and RRRAADSDDDDD, its typical inhibition by low concentrations of heparin, and its unique ability to use both ATP and GTP as cosubstrate. This classification was confirmed by further characterization including (i) enzyme autophosphorylation data that showed two (43 and 40 kDa)catalytic subunits and a 28-kDa noncatalytic subunit, (ii) immunological reaction to the specific human CK2 antibodies, (iii) tryptic peptide maps that resulted in comparable fragmentation of ecto-PK IIα and authentic human CK2α, and (iv) mass spectrometry (MALDI-MS) of HPLC-separated tryptic peptides from ecto-PK IIα and CK2α and microsequencing. The results from comparison with the intracellular CK2 in particular underline the high degree of their homology if not identity.

Many important physiological substrates of CK2 activities point to the physiological significance of CK2 in cellular events (for a recent review see Allende and Allende, Ref. 49). This key role was recently underlined by the major finding that dysregulated expressed catalytic subunit of CK2 acts as an oncogene (50). Two isoforms of CK2 catalytic subunits, α and α′, encoded by two different genes are known to date (51, 52). In addition, a processed CK2α pseudogene (53) and an intronless gene that encodes CK2α (54) have been described. In most tissues the catalytic subunits α and α′ combine with a 28-kDa noncatalytic subunit β, a potent modulator of enzyme activity (55, 56), to form the heterotetrameric holoenzymes α2β2, α′α′β2 or α′2β2. The CK2α/α′ to β ratios may vary considerably (57, 58), and CK2α can also bind to nuclear or cytosolic proteins not related to β (59, 60).

An interesting open question is the mechanism of the transfer of ecto-PK I and ecto-PK II to the cell surface. There are no signal motifs that would indicate a classical secretory pathway through the ER or the Golgi network (47, 61, 62). A further
possibility for cell surface localization would be direct extrusion of the ecto-PKs from cytoplasm to the extracellular space and, on the other hand, up-regulation of ecto-PK shedding, as shown in this study, might represent a mechanism for down-regulation of ecto-PK on the cell surface and, on the other hand, up-regulation of extracellular PK activities.

Acknowledgments—We thank C. Biefer for expert technical assistance and H. Horn and J. Richards for the cell culture work. We are also grateful to Drs. L. Bodenbach and P. Lorenz for providing recombinant enzyme and antibodies, Dr. H. Heid for microsequencing, and Dr. J. Sonka for the suggestion to use PEG for enzyme precipitation. Dr. J. Reed is thanked for discussion and semantic help. We also thank A. Lampe-Gegenheimer for help in manuscript preparation.

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J. Biol. Chem. 1996, 271:111-119.
doi: 10.1074/jbc.271.1.111

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