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Major Cell Surface-Located Protein Substrates of an Ecto-Protein Kinase Are Homologs of Known Nuclear Proteins

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ABSTRACT: Cell surface polypeptides serve as substrates for a casein kinase-like ecto-protein kinase activity which is demonstrable under stringent criteria with intact cells using micromolar levels of extracellular [γ-^{32}P]ATP. Two major ^{32}P-labeled proteins, designated as pp100 and pp120 after their apparent molecular masses on SDS-PAGE under reducing and nonreducing conditions, have repeatedly appeared in the phosphoprotein spectra of different cell types. We have chosen HeLa cells as a source for the biochemical characterization and isolation of pp100 and pp120. Phosphorylation of pp100 and pp120 occurs in their extracellular domains at seryl residues of amino acid side chains. Several criteria deduced from the heparin sensitivity of the ecto-protein kinase and its substrate-induced shedding into the cell supernatant indicated that surface phosphorylation is a function of the ecto-protein kinase. The radioactive phosphorylation of pp100 and pp120 which coincides with their biotinylation on 2D-blots can be reversed by mild trypsination of intact cells. Purification and enrichment of pp100 and pp120 were achieved on the basis of radioactivity detection on and isolation from 1D- and 2D-gels. Amino acid sequence analysis performed on tryptic digests of purified ecto-phosphoproteins in most cases showed significant consensus sequences between pp100 and the nuclear RNA-binding protein nucleolin while pp120 sequences proved to be related to hnRNP U, a nucleoplasmic pre-mRNA-binding protein. Immunochemical analysis using anti-nucleolin and anti-hnRNP U antibodies combined with comparative phosphorylation and characterization of the ecto-proteins with authentic nucleolin and hnRNP U further established the close relationship, suggesting surface membrane versions of the nuclear proteins.

Work in recent years has increasingly emphasized the importance of the cell surface in many phenomena of cell development and cell-environmental interaction in multicellular systems. Full understanding of the surface membrane structure and function remains a major challenge. Much evidence has been accumulated supporting the idea of specific enzyme activities on the cell surface (ecto-enzymes) thought to be integral compounds of the cell membranes with their catalytic side directed outward from the cell. Ecto-enzymes are thus able to metabolize membrane-bound substrates and appropriate extracellular molecules which by size or charge cannot easily penetrate through the intact cell membrane. Therefore, ecto-enzymes are supposed to have a potential for intercellular regulation or reception and transduction of external stimuli. The definition of ecto-enzymes and the criteria by which one might judge whether an enzyme belongs to this category were attempted about 2 decades ago by DePierre and Karnovsky (1974). Our studies of ecto-protein kinase (ecto-PK)[1] activities were based on these early requirements. We have set a series of specific criteria for the detection and analysis of ecto-PK (Kübler et al., 1982a,b, 1983, 1989; Kinzel et al., 1986) which collectively serve to demonstrate and localize the products of the phosphorylation reaction at the cell surface, and to exclude contribution by intracellular substrates in the detectable reaction. These measures include (i) employment of intact cells under rigorous control of their viability monitored by specific stain uptake and release of cytosolic marker enzymes; (ii) use of extracellular ATP, which is unable to penetrate the intact plasma membrane barrier; controls also exclude that radioactive hydrolysis products of [γ-^{32}P]ATP, ^{32}P, or ^{32}PP$_i$, which can enter the cell, participate in the observed phosphorylation; (iii) removal of surface label from the intact cell by trypsin, which by size is limited in its action to the outer plasma membrane; alternatively, trypsin treatment prior to surface phosphorylation should prevent radioactivity incorporation into proteins; (iv) addition of cell lysates to intact cells as well as separating intact from dead cells by fluorescence-activated cell sorting (FACS) to exclude that damaged or dead cells are the source of PK and/or of substrates, and finally phosphorylation of soluble proteins which by their size are limited to the extracellular matrix and which do not harm intact cells; and (v) the selective release of ecto-PK which concomitantly reduces cells' phosphorylation capacity for both exogenous substrates and ecto-proteins.

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[1] Abbreviations: ATP, adenosine 5'-triphosphate; CKII, protein kinase CKII (previously known as casein kinase type II); ecto-PK, ecto-protein kinase; hnRNP U, heterogeneous nuclear ribonucleoprotein U; pp100 and pp120, ecto-phosphoproteins with apparent molecular masses of 100 and 120 kDa, respectively. Enzymes: LDH, lactate dehydrogenase (EC 1.1.1.27); PK, protein kinase (EC 2.7.1.31).

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Studies in our laboratory (Kübler et al., 1982a, 1989) and that of others (e.g., Mastro & Rozengurt, 1976; Chiang et al., 1979; Ehrlich et al., 1986; Fantini et al., 1987; Skubitz & Gueli, 1991; Oda et al., 1991) have shown different types of ecto-PK activities in a wide range of vertebrate cell types. In some studies, ecto-PK activities have been related to a number of biological phenomena (Dey & Majumder, 1987; Friedberg & Kübler, 1990; Myers & Kang, 1990; Naik et al., 1991; Nagashima et al., 1991; Chen & Lo, 1991). One abundant ecto-PK of viable cells has been shown to have the characteristics of a protein kinase CKII (CKII) activity and as such is sensitive to nanomolar levels of heparin and able to utilize ATP and GTP as phosphodonors (Kübler et al., 1982a; Pyerin et al., 1987). Using extracellular ATP, the ecto-PK activity enables phosphorylation of cell surface-located protein substrates (Kübler et al., 1982a,b, 1986), and also soluble external substrate proteins (Kübler et al., 1986, 1987; Šonka et al., 1989). Moreover, ecto-PK can be set free from the intact cell, which may indicate a potential role for cell−environmental interaction over some distance via PK activity (Kübler et al., 1983). Ample evidence for the occurrence of the cosubstrate ATP outside of cells has been published by others [for a review, see El-Moatassim et al. (1992)].

To understand the physiological role of the ecto-PK activity, it is indispensable to search for and analyze its specific substrates and the significance of their phosphorylation and dephosphorylation. The cell surface protein phosphorylation pattern obtained with intact cells presumably represents natural products of the ecto-PK reaction. However, no identification or direct analysis of surface phosphoproteins has yet been reported.

In this study, the purification and identification of cell surface substrates for ecto-PK have been attempted. We report here results of studies with HeLa cells as a general model that identify two major ecto-phosphoproteins, designated as pp100 and pp120. Our data show a close relation of pp100 and pp120 to certain nuclear proteins.

EXPERIMENTAL PROCEDURES

Cell Cultures. HeLa monolayer cells were grown in minimum essential medium, 10% calf serum (Gibco-BRL). Alternatively, HeLa monolayer cells were adopted to and cultivated in serum-free medium (HL-1 from Ventrex) for an as yet unlimited number of passages (>90). The suspension form HeLa-S3 was grown in Joklik's medium, 5% calf serum. Cells were propagated free from mycoplasms. Optimal cultures were treated under the isoosmotic conditions described under Phosphorylation Conditions or as indicated in the particular experiment. For experiments, HeLa cultures were routinely used at densities of 5×10^5 monolayer cells per 5 cm plate or 1×10^6 suspension cells/mL.

Other cell types employed for the comparative experiments analyzing cell surface phosphoprotein spectra were as follows: human embryo lung (HEL); human vulva carcinoma (A 431); human B-lymphoblasts (BJAB); human osteosarcoma cells (MG 63); rabbit kidney cells (RK 13); mouse fibroblasts (3T3 and virally transformed counterparts SR 3T3); rat liver cells (C I); rat pheocytochroma cells (PC 12); chicken embryonal cells (CEC and Rous sarcoma virus-transformed CEC). For cultivation of the individual cell types, their appropriate growth conditions were routinely applied.

Cell Viability. Control of cell intactness and cell membrane status under phosphorylation conditions was as previously described (Kübler et al., 1989). Briefly, cell morphology was assessed by light microscopy; membrane permeability by uptake of the fluorescent dye ethidium bromide which stains susceptible cells instantly. For control of efflux, the occurrence in extracellular fluid of the cytosolic enzyme lactate dehydrogenase was measured.

Antisera and Purified Protein Samples. Purified human nucleolin was from Dr. M. Caizergues-Ferrer and Dr. F. Amalric, Toulouse; anti-nucleolin was from Dr. F. A. Anderer, Tübingen; anti-hnRNP U (3G6), anti-hnRNP C1/C2 (4F4), and purified recombinant hnRNP U were from Dr. S. Piñol-Roma and Dr. G. Dreyfuss, Philadephia; recombinant CKII subunits (α and β) were from L. Bodenbach and Dr. W. Pyerin, Heidelberg; and anti-LBP 110 was from Dr. H. K. Kleinman, Bethesda.

Phosphorylation Conditions. Protein phosphorylation by ecto-protein kinase activity on intact cells was routinely carried out as detailed earlier (Kübler et al., 1982a). Briefly, washing and incubation of cells were done with prewarmed assay buffer consisting of 70 mM sodium chloride, 30 mM tris(hydroxymethyl)aminomethane, 5 mM magnesium acetate, 0.5 mM EDTA, 5 mM potassium phosphate, and 75 mM glucose (pH 7.2; osmolarity: 290 ± 10 mOsm). The labeling of prewashed cells was initiated by addition of 12.5 μCi/mL [γ-^{32}P]ATP (0.5 μM final ATP concentration) and allowed to proceed for 15 min at 37 °C. The reaction was stopped by removal of the cell supernatant and two washes with 5 mL portions of ice-cold assay buffer containing 1 mM unlabeled ATP. Washed cells were immediately lysed with sample buffer containing 2.5% SDS. Proteins from the supernatant were precipitated by addition of 5 volumes of methanol in the presence of 1 mg of bovine serum albumin/mL for 5 h at −20 °C. Radioactivity incorporation into cellular proteins was analyzed by SDS−PAGE (8% or 9−15% acrylamide gradient) and autoradiography. Where necessary, surface protein phosphorylation of intact cells was carried out in the presence of 10−100 μg/mL heparin (pig mucosa lipo-hepin; 5000 IU/mg from Riker-Kettelhak). For control, ^{32}P-labeled cells were treated with heparin after surface phosphorylation.

For phosphorylation of nucleolin and hnRNP U (0.5 mg of either protein), the purified nuclear proteins were incubated for 15 min at 37 °C in 200 μL of assay buffer (as above) and 0.5 μM [γ-^{32}P]ATP either with 2×10^5 monolayer cells or with recombinant CKII subunits (0.1 mg of α and 0.3 mg of β). Cell supernatants were centrifuged 5 min at 100g to remove cells and a further 5 min at 14000g. CKII activity was terminated by addition of 1 mM unlabeled ATP and 0.05% SDS.

Release of Ecto-PK Activity by Iterative Incubation with Exogenous Substrate. For ecto-PK depletion of intact cells, the method of substrate-induced shedding was used as detailed by us earlier (Kübler et al., 1983). Briefly, subconfluent monolayer cell cultures were washed twice with (37 °C) prewarmed incubation solution as given under Phosphorylation Conditions prior to incubation with the same solution including 1 mg/mL phosvitin. After 5 min of moving the mixture gently over the cells, the supernatant containing PK activity was aspirated, and the cells were provided with fresh phosvitin solution for another 5 min.

Surface enzyme-depleted cells were tested for their ^{32}P-labeled surface phosphoprotein spectra as described above. It is important to note that with HeLa cells each wash with phosvitin resulted in decreased surface protein labeling intensities up to the fourth wash where practically no label was generated (Kübler et al., 1983).

Cell Surface Biotinylation. For biotinylation with *N*-hydroxysuccinimide−biotin (NHS−biotin, obtained from Fluka), the method given by Cole et al. (1987) was used. Briefly, NHS−biotin was freshly prepared from a 10 mg/mL stock solution in DMSO and used at 0.02 mg/mL for labeling of prewashed cells. After 15 min incubation, the reaction was stopped by removal of the cell supernatant fluid and reincubation for 10 min with isoosmotic assay buffer containing 1 mM ethanolamine. Finally, the cells were washed once with ethanolamine-containing buffer and twice with assay buffer alone. The cell proteins 2D-separated by isoelectric focusing (IEF) and SDS−PAGE were Western-blotted to a PDVF membrane (Immobilon-P) and stained with peroxidase-coupled streptavidin.

Protein cross-linking of cell surface proteins was carried out as previously described (Jordan & Kübler, 1992) with dithiobis(succinimidyl propionate) (DSP) and sulfosuccinimidyl 2-(*m*-azido-*o*-nitrobenzamido)ethyl-1,3′-dithiopropionate (SAND) (from Pierce). Briefly, DSP was freshly prepared as a 50 mM stock solution in dimethyl sulfoxide. SAND was dissolved in water and kept dark as a 100 mM stock solution. Cells were treated for 10 min at room temperature with cross-linkers in isoosmotic Tris-containing assay buffer (as given under "Phosphorylation Conditions") but lacking Tris. The reaction with intact cells was quenched by three washes with isoosmotic assay buffer containing 35 mM Tris.

Other Enzymatic Treatments of Intact Cells. Trypsin treatment of cells was carried out by incubation with 0.01% trypsin in isoosmotic assay buffer (as described under "Phosphorylation Conditions") for 4 min at 37 °C. Termination was by addition of 0.5% soybean trypsin inhibitor and two washes in the isoosmotic assay buffer.

Cell Lysis by Triton X-100. HeLa-S3 (1×10^6 cells/mL of extraction solution) or monolayer cells were extracted with different Triton X-100 concentrations in 50 mM Tris-HCl buffer, pH 7.4 (including 250 mM NaCl and inhibitor cocktails as given below). The Triton X-100-insoluble fraction was separated by centrifugation at 1000*g* for 5 min.

The extraction solution contained protease and phosphatase inhibitors as follows. The protease inhibitors included 0.5 mM phenylmethanesulfonyl fluoride (PMSF), 0.5 mg/L leupeptin, 0.5 mg/L pepstatin A, 0.5 mg/L 1-[N^{α}-(*trans*-epoxysuccinyl)-L-leucylamido]-4-guanidinobutane (E 64), 1 mM 1,10-phenanthroline (all prepared as a 100× stock solution in ethanol), 0.5 mg/L aprotinin, 2 mM EDTA, 2 mM EGTA, 5 mg/L soybean trypsin inhibitor, and 0.5 mM aminohexanoic acid (all prepared as a 100× stock solution in water); the cocktail of phosphatase inhibitors included 10 mM NaF, 0.25 mM sodium vanadate, 0.25 mM sodium molybdate, 5 mM β-glycerophosphate, and 10 mM α-naphthylphosphate.

Purification Conditions for pp100 and pp120. HeLa-S3 suspension cells (density 3×10^5/mL) were washed and ecto-proteins phosphorylated for 15 min with 2 nmol of unlabeled ATP/10^6 cells as described under "Phosphorylation Conditions". Cells were then resuspended at 5×10^7/mL in extraction solution and disrupted by sonication as described above. The sonicates were centrifuged at 160000*g* for 45 min, and the particulate fraction was stored at −70 °C. Material equivalent to 2×10^9 cells was resuspended in 15 mL of sonification buffer with the help of a homogenizer (Ultra-turrax) before mixing with 1 volume of sample buffer (5% SDS). Nucleic acids were removed by digestion with 5000 units of Benzon-DNase (Merck)/5 mM $MgCl_2$ for 15 min. Lysates were heated to 54 °C prior to electrophoretic separation. Preparative one-dimensional SDS−PAGE was in 7.5% acrylamide slab gels under reducing conditions. Samples equivalent to 4×10^7 cells per gel were separated at 13 mA for 16 h. A prestained 116 kDa marker protein as well as radioactive cell lysate was added to survey migration or excision, respectively. Subsequently, the gels were stained for 20 min in freshly prepared aqueous 0.2% Coomassie-Brilliant Blue R-250 and destained in water, and protein bands were excised over a light box. The excised gel slices were electroeluted according to the procedure described by Hunkapillar et al. (1983). Efficiency was determined by radioactively labeled pp100 and pp120. Material equivalent to 8×10^7 cells was applied per elution unit, equilibrated for 3 h in 200 mM Tris−acetate, pH 7.8, 2% SDS, and 10 mM DTT, and proteins were eluted from the acrylamide for 16 h at 60 V in 50 mM Tris−acetate, 0.2% SDS, and 1 mM DTT. The eluate was precipitated at −20 °C with 5 volumes of methanol, and the precipitates were collected by centrifugation into a single test-tube. The collected precipitates were subjected to 2D-separation by isoelectric focusing (IEF) in the first dimension and SDS−PAGE in the second dimension. Samples were solubilized in 500 μL of IEF sample buffer, and material isolated from 2.5×10^8 cells per IEF-gel was separated in the presence of radioactively labeled ecto-proteins electroeluted from 2×10^7 cells. Ampholytes (Pharmacia-LKB) were 1% pH 3.5−10, 2% pH 4−6, and 2% pH 5−7. After SDS−PAGE (cf. above) in the second dimension, the gels were fixed in 20% methanol/10% acetic acid, stained 1 h with 0.1% Coomassie, and destained for 3 h. The identified protein spots were excised, washed extensively with water, chopped, and washed twice for 60 min at 37 °C in 0.1 M $(NH_4)HCO_3$, pH 8.5 (digestion buffer). Gel pieces were partially dehydrated under vacuum for 30 min in a speed-vac, then covered to the volume of their hydrated state with trypsin solution (2.5 μg/mL in digestion buffer), and incubated overnight at 37 °C. The gel pieces were washed with buffer and then with acetonitrile, and the peptide-containing supernatants were frozen and concentrated in a speed-vac.

Separation and Sequencing of Tryptic Peptides. The peptide samples obtained after IEF/SDS−PAGE and tryptic digestion were adjusted to 5% acetonitrile/20% trifluoroacetic acid (TFA), left 30 min for degassing and loaded onto a reversed-phase HPLC column (C18, Aquapore OD-300 618-222; 22 × 0.21 cm; 7 μm; Applied Biosystems), washed with 0.1% TFA, and eluted in a gradient toward 80% acetonitrile/0.085% TFA over 100 min at 100 μL/min and approximately 70 bar. Elution was monitored by the absorption at 220 nm. Selected peptides were sequenced with the 477A protein sequencer, Applied Biosystems, with the on-line HPLC-system 130A. Sequencing sensitivity was further increased through a 100 μL injection loop. Computer analysis and sequence comparisons were performed using the special program package (HUSAR) developed in the Department of Molecular Biophysics, German Cancer Research Center.

Phosphopeptide Mapping. Radioactively labeled cell surface proteins pp100 and pp120 were isolated from 10^7 HeLa-S3 cells by electroelution as given above. Phosphorylation of the purified nuclear proteins nucleolin and hnRNP U was achieved as detailed under "Phosphorylation Conditions". The samples were adjusted to identical buffer conditions and then split in three portions. One portion was denatured immediately with 1 volume of sample buffer (5% SDS), and the others were digested at 37 °C by addition of either 1 μg of V8-protease or chymotrypsin. Proteolysis was terminated after 10 and 60 min by mixing with 1 volume SDS sample buffer and boiling. Phosphopeptides were separated by SDS−PAGE (12% acrylamide) and analyzed by autoradiography.

Phosphorylated Amino Acid Analysis. Identification of the phosphorylated amino acid residue in pp100 and pp120 was performed by observing radioactive bands after partial hydrolysis in 6 N HCl (110 °C for 6 h) and 2D high-voltage electrophoresis at pH 1.9 in the first dimension and pH 3.5 in the second dimension on thin-layer cellulose plates as described earlier (Kübler & Barnekow, 1986).

Immunological Methods. For *immunostaining* of proteins after Western blotting, the membrane was first soaked in Tris-buffered saline (TBS) with 3% BSA for 1 h, followed by incubation for 2 h with antibodies in TBS/0.2% Tween 20 (TBS/T). The blot was then washed 3 times in TBS/T and incubated with peroxidase-coupled secondary antibody for 2 h. After another wash, the blot was finally stained with 50 mM Tris-HCl, pH 7.4, with 0.03% 4-chloronaphthole, 0.03% hydrogen peroxide, and 0.001% redox enhancer (100 mg/mL *p*-phenylenediamine, 50 mg/mL sodium metabisulfite).

For immunoprecipitation of nucleolin and pp100, 5×10^5 cells were lysed in 50 μL of 50 mM Tris-HCl, pH 7.4, 1% SDS, and 100 mM DTT, boiled, and diluted 10-fold with TBS, 1% deoxycholate, 0.5% TX-100, and the inhibitor cocktails (s.a.). The extract was centrifuged at 14000*g*, the supernatant was incubated for 2 h with anti-nucleolin (1:100), and the complexes were precipitated by addition for 2 h of 5 mg of prewashed protein A−Sepharose. The beads were sedimented at 1000*g*, washed with dilution buffer and then with Tris-HCl, pH 7.4, and finally extracted by boiling in SDS sample buffer. The nonprecipitated proteins were precipitated from the supernatant with 5 volumes of methanol. Samples were separated by SDS−PAGE, blotted to Immobilon-P (Millipore), and immunostained with anti-nucleolin (1:500).

Immunoprecipitation of hnRNP U (Dr. G. Dreyfuss, personal communication) followed a procedure with 5×10^5 cells lysed in 1% Empigen BB-detergent (in 50 mM Tris-HCl, pH 7.4) and precipitated with the hnRNP U antibody (3G6) and anti-hnRNP C1/C2 (4F4), each 1:100, and detected by immunoblotting with these antibodies (1:1000).

Indirect immunofluorescence microscopy was routinely carried out with cells grown on cover slips as follows: cells were fixed with 2% paraformaldehyde/PBS (pH 7.4) and either permeabilized with acetone (5 min at −20 °C) or methanol (10 min at −20 °C), or directly used for antibody staining with anti-pp105/nucleolin (1:250) or anti-hnRNP U (3G6; 1:500). Staining of the primary antibodies was done with dichlorotriazinylaminofluoresceine (DTAF)-coupled goat anti-rabbit antibodies (1:200), and fluorescence was analyzed with a Zeiss Axiophot microscope.

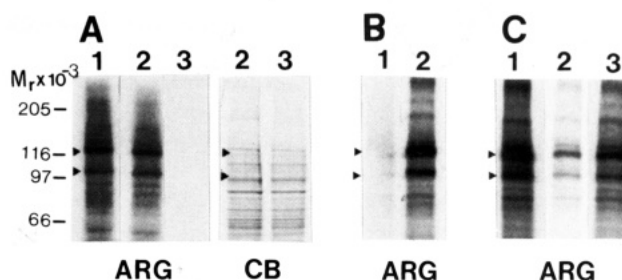

FIGURE 1: Major ^{32}P-labeled proteins as generated by ecto-protein kinase of HeLa cells. Optimal HeLa cultures as detailed under Experimental Procedures were phosphorylated for 15 min. Labeled cells were rinsed and lysed with SDS sample buffer, and the total cell lysates (from 0.5×10^6 cells) were electrophoresed by an SDS−PAGE (8−15%) polyacrylamide gradient. Shown are autoradiographic patterns (ARG) and Coomassie (CB)-stained proteins of (A) (lane 1) cells grown in MEM with 10% calf serum, (lanes 2) cells grown in serum-free HL-1 medium, and (lanes 3) ^{32}P-labeled cells after treatment with 0.05% trypsin for 3 min. The autoradiograms of (B) show (lane 1) cells treated for 10 min with heparin-containing assay mixture (see Experimental Procedures) prior to radioactive surface phosphorylation (identical results were obtained when heparin was present during the phosphorylation reaction) and (lane 2) cell treatment with heparin as in lane 1 but after radioactive surface phosphorylation. The autoradiograms of (C) show (lane 1) cells after normal radioactive surface phosphorylation (control), (lane 2) cells treated for phosvitin-induced ecto-PK depletion (see Experimental Procedures) prior to radioactive surface phosphorylation, and (lane 3) cells treated with phosvitin as in lane 2 but after radioactive surface phosphorylation. The mobility of marker proteins is given on the left. The positions of pp100 and pp120 are marked; shown are only the relevant parts of the analysis.

RESULTS

Detection and Properties of the Cell Surface Phosphoproteins pp100 and pp120. The phosphorylation of cell surface proteins by ecto-PK can be assessed with intact cells (under the specific criteria denoted in the introductory part of this paper) by the covalent and time-dependent incorporation of ^{32}P radioactivity from exogenous [γ-^{32}P]ATP or, with about 50% efficiency, from exogenous [γ-^{32}P]GTP (Kübler et al., 1982). Cells from different origin (as indicated under Experimental Procedures) exhibit cell-type specific phosphoprotein spectra, however, generally including two major substrates, termed pp100 and pp120 as indicated in Figure 1. This study concentrates on the identification of the phosphoproteins pp100 and pp120 using HeLa cells as a model.

pp100 and pp120 phosphorylation with HeLa cells grown in 10% calf serum or grown serum-free in chemically defined medium was identical (Figure 1A). This result excludes the possibility of contamination by serum proteins, and therefore these phosphoproteins are considered to be genuine cellular proteins.

Upon treatment of phosphorylated cells with 0.05% trypsin for 3 min, a condition used in the cell culture routine, the radioactive label can be completely removed from intact cells (Figure 1A, lane 3) while, on the other hand, the proteolytic attack did not bring about any change of Coomassie staining of total cell proteins, a result which is compatible with the surface location of the ^{32}P label.

In a previous study, this ecto-PK was related to casein kinase (CK) activity having a known sensitivity to low concentrations of heparin (Pyerin et al., 1987). Heparin was used here to specify the pp100 and pp120 phosphorylation. When cells were incubated with heparin before or during the surface phosphorylation assay, the radioactive labeling

was totally erased as shown in Figure 1B, lane 1. However, heparin treatment of cells after surface protein phosphorylation did not affect the ^{32}P-labeling intensity as compared to untreated control cells (Figure 1B, lane 2). Hence, this result indicates that pp100 and pp120 phosphorylation is likely mediated by ecto-PK (CK) activity. For support, it is of equal importance to note that the ecto-PK can be released from intact cells into their supernatant fluid through specific interaction with exogenous substrate protein (Kübler et al., 1983). Thus, a single incubation of intact cells with phosvitin drastically diminished the cells' capacity to phosphorylate their surface proteins as shown in Figure 1C, lane 2. Such a decrease could be correlated with up to four cell washes with phosvitin and the subsequent appearance of ecto-PK in the supernatant (Kübler et al., 1983). The autoradiogram in Figure 1C, lane 3, indicates that the surface phosphoproteins pp100 and pp120 appeared not to be dissociated from the cell surface when phosvitin treatment was carried out with ^{32}P-phosphorylated cells, which is in clear contrast to the ecto-PK release. In addition, cell washes did not contain ^{32}P-labeled proteins (data not shown). The observation that treatments with phosvitin did not remove pp100 and pp120 from the cells but decreased the degree of their phosphorylation upon incubation with [γ-^{32}P]ATP argues against the possibility of an "autophosphorylation" of these proteins. In summary, considering the degree of specificity and the availability of the CK-like enzyme, we postulate that phosphorylation of pp100 and pp120, the two major surface substrates of HeLa cells, is a function of ecto-PK activity.

The electrophoretic separation of cell proteins on polyacrylamide gels in the presence of SDS (SDS−PAGE) under reducing and nonreducing conditions led to essentially the same phosphoprotein pattern as in Figure 1. Thus, disulfide bonds appear to be excluded, and the two phosphoproteins pp100 and pp120 presumably occur as protein monomers. This is supported by data after treatment of intact cells with mild cross-linking agents of the succinimidyl type (DSP, SAND) which showed that pp100 and pp120 were not affected. Such results suggest that pp100 and pp120 occur as solitary proteins and, particularly, are not closely associated with each other at the cell surface (data not shown).

To identify pp100 and pp120 specifically among total HeLa cell proteins, we applied two-dimensional (2D) gel electrophoresis by isoelectric focusing (IEF) and SDS−PAGE. A typical radioactive 2D pattern is shown in Figure 2B, indicating the pp100 radioactivity as a single spot while pp120 was resolved in a slightly broader pH range and, in addition, that only a small portion of total pp120 enters the IEF-gel, as was repeatedly observed. On the other hand, the barely detectable protein staining of both phosphoproteins in Figure 2A indicates high specific activities. In order to identify the phosphorylated amino acid residues in pp100 and pp120, their radioactive 2D-spots were subjected to partial acid hydrolysis and high-voltage electrophoresis. Both phosphoproteins revealed that phosphorylation occurred at Ser residues (not shown).

2D separation of cell proteins was used for the comparison of surface-labeled cells which have been double-labeled by radioactive surface phosphorylation and subsequent biotinylation with NHS−biotin (for details, see Experimental Procedures). Both labels colocalized with pp100 and pp120, and both labels were similarly sensitive to treatment of intact cells with trypsin known to be limited in its action to the outside of cells (Figure 3). This result represents an additional and independent support for the surface location of these phosphoproteins.

The type of association of pp100 and pp120 with the cell membrane can be described in an operational manner. It became evident that the proteins were not detached from the intact cells through repeated washes with buffers at physiological salt concentration. The use of high-salt buffers (thought to help discern peripheral from integral membrane proteins) (Findlay, 1990) appeared to be disadvantageous in studies with intact cells since such conditions will automatically destroy the cell integrity. On the other hand, when surface ^{32}P-labeled cells were sonicated and the pelleted material was extracted with up to 1.5 M NaCl, we found practically all radioactive pp100 and pp120 in the 100000g pellet and none in its supernatant (not shown). In addition, extraction experiments revealed that both ^{32}P-labeled pp100 and pp120 could be readily solubilized through treatment with nonionic detergent (Triton X-100) at concentrations where the extent of general protein solubilization was comparably low as indicated by the strength of Coomassie stain (see Figure 7 below). Such extraction behavior of pp100 and pp120 was presumed compatible with a tight association with the cell surface membrane.

Isolation and Microsequencing of pp100 and pp120. As detection of the incorporated radioactive label was required in order to follow the isolation of pp100 and pp120, we developed a protocol based on rapid inactivation by SDS of proteolytic and dephosphorylating enzymes as described under Experimental Procedures. The isolation procedure was monitored through small amounts of radioactively labeled surface proteins. To correct for any physical differences arising from this method (e.g., band-shift), the bulk protein for isolation was also phosphorylated with unlabeled ATP (see Experimental Procedures). Isolation began with a first enrichment by SDS−PAGE. In this step, the removal of nucleic acids by an SDS-resistant nuclease was necessary for optimum protein resolution during electrophoretic separation. In the second step, about 90% of the phosphoproteins were recovered by electroelution from nonfixed acrylamide gel pieces. Protein samples were then further concentrated and freed from SDS through precipitation in methanol. This regimen allowed the stepwise collection of the phosphoproteins in order to obtain sufficient quantities for microsequencing. In the final step, pp100 and pp120 were purified by 2D-gel electrophoresis (IEF/SDS−PAGE as above). A separation of the isolated protein fractions is shown in Figure 4. On the basis of a starting sample of 2×10^9 HeLa cells which resulted in the purification of about 6 μg of pp100 and 4 μg of pp120 as estimated from the intensity of the Coomassie staining of the spots on 2D-gels, we calculated that about 10^4 copies of pp100 and pp120 per cell exist, which underlines the presumably high specific activities of both phosphoproteins indicated by Figure 2.

To carry out microsequencing of pp100 and pp120, the proteins were identified by radioactivity, excised from the gel, and digested with trypsin. Tryptic peptides were separated on a reversed-phase HPLC column and subjected to automated sequencing. Peptide sequence data aligned to protein sequences from data bases included in the program package HUSAR (see Experimental Procedures) are given in Table 1. These showed significant sequence identity between pp100 and nucleolin, a major nucleolar ribosomal RNA-binding protein. In addition, most peptides of pp120 were identical to the heterogeneous nuclear ribonucleoprotein

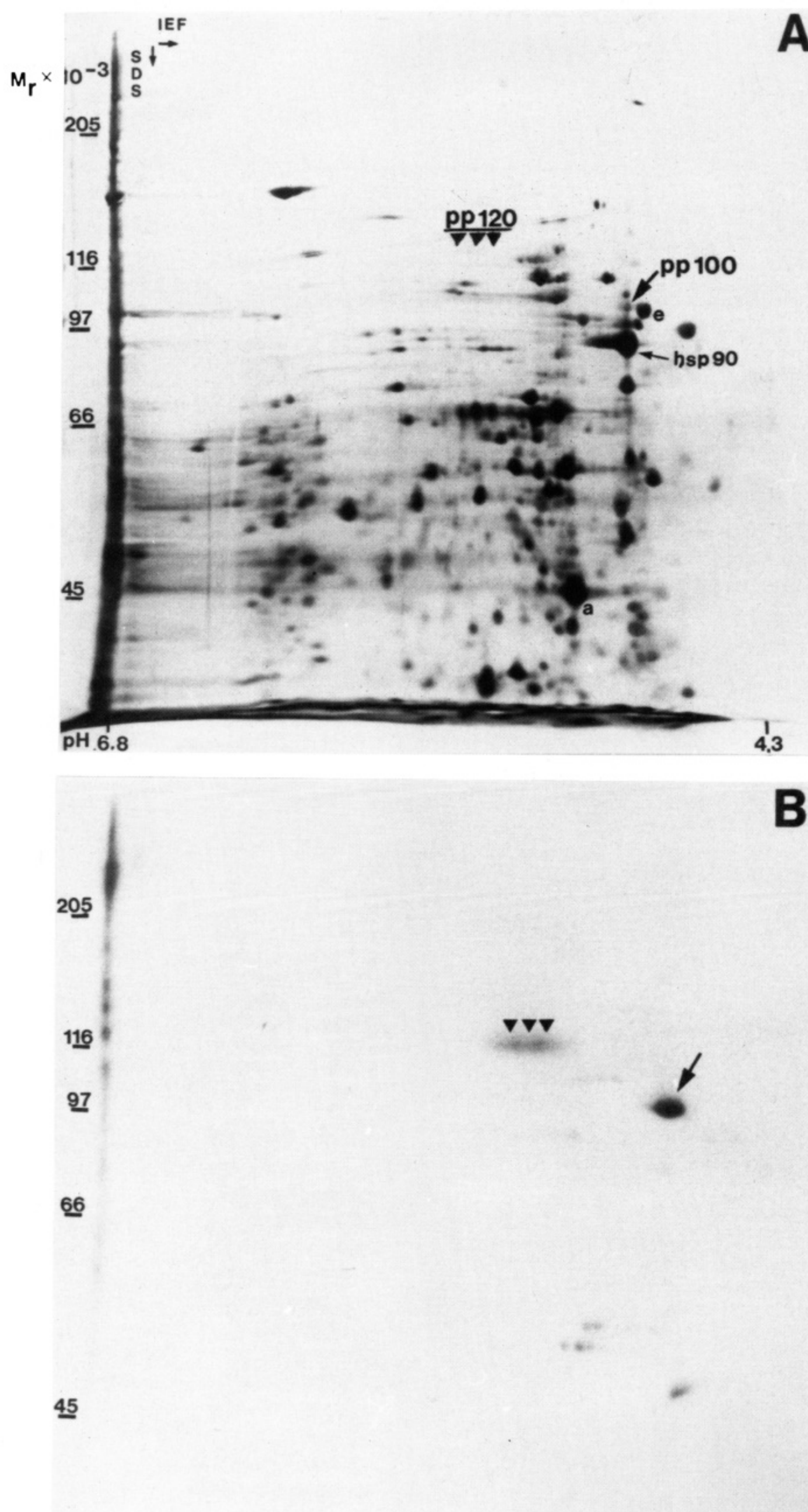

FIGURE 2: Phosphoprotein image of a 2D-gel of cell surface proteins resolved by IEF and SDS−PAGE. Phosphorylated HeLa cells were lysed, and material equivalent to approximately 10^6 cells was subjected to isoelectric focusing (IEF) followed by SDS−PAGE (8% polyacrylamide gel). Shown are the pattern of (A) the silver-stained gel and (B) the corresponding autoradiography. The positions of pp100 and pp120 and some known proteins deduced from the AMA master database (Celis et al., 1990) [marked as *e*, endoplasmin; *hsp90*, heat shock protein 90; *a*, actin] are indicated.

U (hnRNP U), a nucleoplasmic pre-messenger RNA-binding protein. In contrast, certain peptide sequences were obtained for both pp100 and pp120 (not shown) that could not be clearly determined, possibly due to mixtures of tryptic

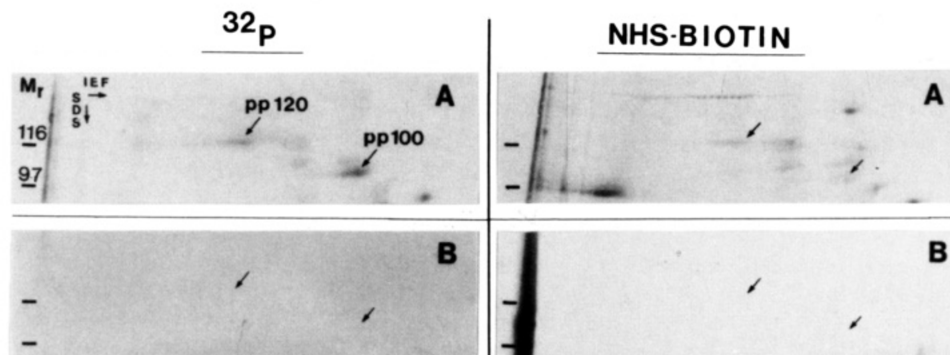

FIGURE 3: Comigration of surface ^{32}P-phosphorylated and biotinylated pp100 and pp120 in 2D-gels (IEF/SDS−PAGE) and sensitivity of the labels to trypsin treatment of intact cells. HeLa cells were subsequently surface-labeled by NHS−biotin as described under Experimental Procedures, and after washing with isoosmotic assay buffer subjected to radioactive surface phosphorylation. The trypsin treatment of labeled cells was carried out as in Figure 1A, lane 3. After the cell lysates were resolved by 2D-gel electrophoresis as in Figure 2, the material was transferred to a PDVF membrane and stained with peroxidase-coupled streptavidin. Shown are the results by comparison of (A) the two types of surface labels and (B) their sensitivity toward treatment of intact cells with trypsin. The positions of pp100 and pp120 are marked.

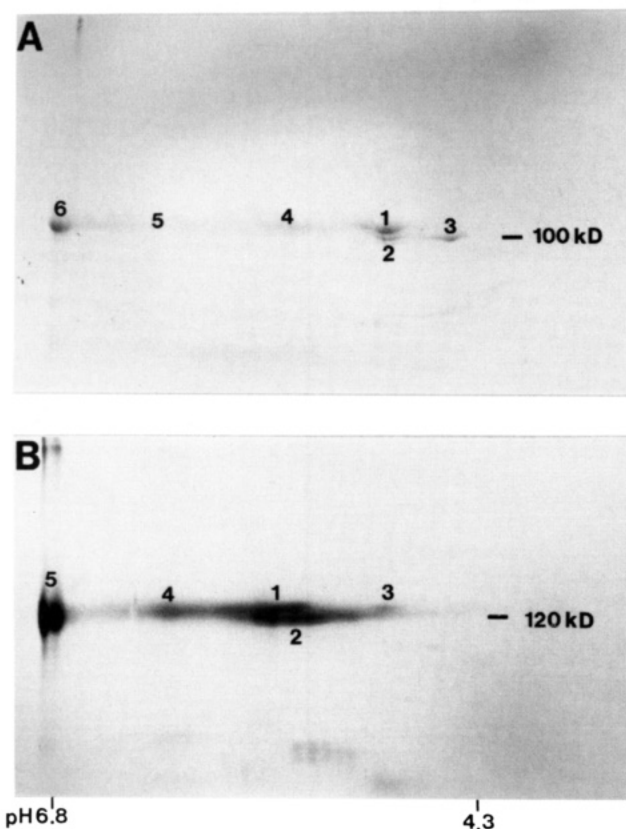

FIGURE 4: 2D-gel electrophoresis of isolated and enriched pp100 and pp120 preparations. Nonradioactively phosphorylated HeLa S3 cells were lysed and batchwise-mixed with 1% of radioactively phosphorylated material in order to survey 2D-gel electrophoresis as described in the legend of Figure 2. The pp100 and pp120 spots identified by autoradiography were excised from the gel and recovered by electroelution. The pooled phosphoproteins from a total of 2×10^9 cells were reelectrophoresed by the 2D-gel system. Shown are Coomassie-stained gels of (A) pp100 and (B) pp120 preparations. Stained spots defined by numbers as given in the figure were excised from the gel, and radioactivity was determined by liquid scintillation counting. Approximately 80% of the measured radioactivity was found in pp100 spots 1 and 2; in the case of pp120, spots 1 and 2 contained about 65% and spot 5 (not resolved by IEF) contained about 25% of the radioactivity.

peptides present in these fractions that were not resolved by reversed-phase HPLC. It was not possible to match such sequences either with the nuclear proteins or with trypsin used for peptide fragmentation.

Table 1: Amino Acid Sequences of Peptides Obtained from the Phosphoproteins pp100 and pp120[a]

polypeptide	sequence determined	amino acid position in nucleolin
pp100	NVAEDE	231−237
	FGYVDFESAEDLEK	349−362
	NLPYK	399−403
	SISL	458−461
	GGXGGF	668−673
polypeptide	**sequence determined**	**amino acid position in hnRNP U**
pp120	XXXPVN	2−7
	GYFEYIEENK	237−245
	EKPYFPIPEEYXXI	444−458

[a] Polypeptides pp100 and pp120 from the total lysate of HeLa cells, enriched by 1D PAGE, separated after 2D-gel electrophoresis, and identified by radioactivity (see Experimental Procedures), were excised from polyacrylamide gels and digested with trypsin, and the tryptic peptides were sequenced. About 6 μg of pp100 (60 pmol) and 4 μg of pp120 (30 pmol) were purified from a starting sample of 2×10^9 HeLa cells. Shown are peptide sequences which could be aligned to the sequence of human nucleolin (in the case of pp100) and hnRNP U protein (in the case of pp120). The numbers indicate the corresponding amino acid positions within the nucleolin and hnRNP U protein sequences, respectively. X, unidentified amino acid. The initial yields of the sequencing reaction were in the range of 3−10 pmol.

pp100 and pp120 Relation to Nucleolin and hnRNP U. Since the surface phosphoproteins account only for a small fraction of the cell proteins (as shown above in Figure 2), it was necessary to consider the possibility that the observed results from microsequencing (Table 1) are affected by comigrating surface phosphoproteins. Therefore, we attempted to exclude this possibility by employing specific antibodies to nucleolin and hnRNP U. In a first approach using cell lysate from surface-phosphorylated HeLa cells, it was found (Figure 5) that radioactive pp100 and pp120 can be immunoprecipitated by the specific antibodies and run identically in SDS−PAGE with purified nucleolin and recombinant hnRNP U stain, respectively. In a second assay using immunostaining after 1D- and 2D-gel blotting onto nitrocellulose paper, the identity of the pp100 and pp120 labels with the anti-nucleolin and anti-hnRNP U signals was confirmed (not shown). In addition to the results described above, these antibody analyses indicate that pp100 and pp120 represent at least closely related, if not identical, isoforms to their known nuclear counterparts.

Anti-nucleolin and anti-hnRNP U antibodies which recognize pp100 and pp120 enriched in blots (as shown above

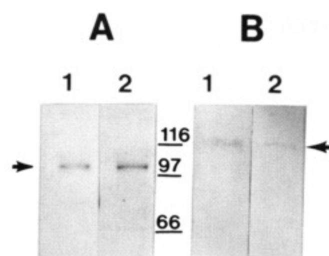

FIGURE 5: Immunoprecipitation of ecto-phosphoproteins pp100 and pp120 with antinucleolin and anti-hnRNP U antibodies. Lysates from 5×10^5 phosphorylated HeLa S3 cells were immunoprecipitated with anti-nucleolin (1:100) or anti-hnRNP U (1:100) as described under Experimental Procedures. The immunoprecipitates were separated by SDS−PAGE (8% acrylamide) and blotted to Immobilon-P (250 mA for 2.5 h). The blots were stained with either anti-nucleolin (1:500) or anti-hnRNP U (1:1000), respectively. Shown are the results of immunoprecipitations (A, lane 1) with antinucleolin and (B, lane 1) with anti-hnRNP U. Lanes 2 in (A) and (B) show autoradiography. The mobility of molecular weight marker proteins is given on the right. Shown are only the relevant parts of the analysis.

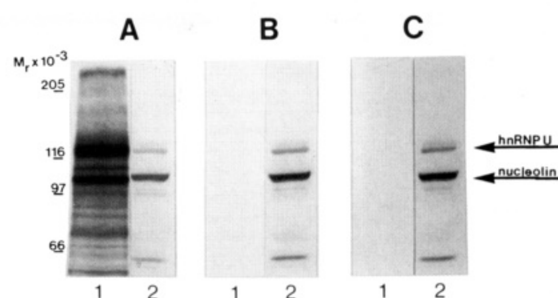

FIGURE 6: Comparison of the ecto-phosphoproteins pp100 and pp120 with immunostaining of nucleolin and hnRNP U after trypsin treatment of intact cells. (A) HeLa cells were radioactively surface-phosphorylated, and material from 0.5×10^6 labeled cells was subsequently separated by SDS−PAGE (8% acrylamide) and Western-blotted to Immobilon-P. Alternatively, HeLa cells were treated (B) prior or (C) with 0.05% trypsin solution for 3 min after surface ^{32}P-phosphorylation. Immunostaining of the blots with anti-nucleoilin and anti-hnRNP U was carried out as described under Experimental Procedures. The results in panels A, B, and C are shown by comparison of (lanes 1) autoradiograms and (lanes 2) immunostains of Western blots.

in Figure 5) did not help to provide direct evidence of the cell surface location of the phosphoproteins by indirect immunofluorescence microscopy. We used nonpermeabilized (intact) and Triton X-100-permeabilized cells to test the antibodies available. However, in no case could we detect pp100 or pp120 surface fluorescence above the background of control cells, while permeabilized cells showed significant nuclear staining (data not shown). The failure to immunostain pp100 and pp120 on the intact cells may be due to the low number of copies (on the order of 10^4 per cell were determined; see above) which is well below the sensitivity of this technique.

Different evidence for the surface location of pp100 and pp120 was approached. One approach is related to the fact that trypsin removes the cell surface label from intact phosphorylated and surface biotinylated cells as described above (see Figure 1A and Figure 3). In contrast, as demonstrable with specific antibodies by the immunoblotting technique, the protein levels of nucleolin and hnRNP U appeared unchanged, indicating that the trypsin action was indeed exclusively directed toward the cell surface proteins (Figure 6). Another line of evidence comes from the different subcellular location of pp100 and pp120 versus nucleolin and hnRNP U, on the cell surface and in the nucleus. It relates to a presumably different behavior under mild detergent extraction conditions where the bulk of proteins including nuclear proteins remains insoluble as shown in Figure 7B. The Triton X-100 extraction experiments with radioactively phosphorylated cells include analysis of the cell extracts for labeled pp100 and pp120, and reactivity toward antinucleolin and anti-hnRNP U antibodies. We consistently found a similar high solubility for both ^{32}P-labeled pp100 and pp120 within the Triton X-100 concentration range tested (Figure 7A) while major solubilization of nucleolin and hnRNP U occurred only at the higher (1%) detergent concentration as indicated by the strength of their immunostains (Figure 7C).

Previous results by Belenguer et al. (1990) have shown genuine nucleolin to be a substrate of CKII activity, and similarly that hnRNP U (Choi & Dreyfuss, 1984) is a phosphoprotein. To extend characterization of the surface and their nuclear protein forms, we performed comparative experiments to study their phosphorylation by the CKII-like

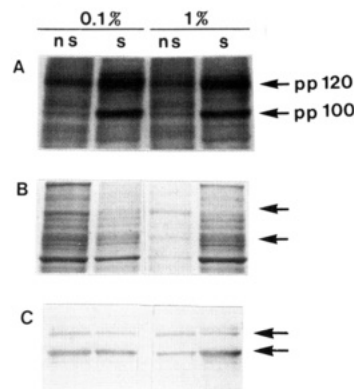

FIGURE 7: Phosphorylation of ecto-PK substrates pp100 and pp120 and effects of Triton X-100 on their solubilization. HeLa cells were surface-phosphorylated and labeled cells subsequently lysed with 0.05−1% Triton X-100 [in 50 mM Tris-HCl (pH 7.4)/250 mM NaCl] as described under Experimental Procedures. Cell treatment with buffer alone in the absence of detergent served as a control. Cell lysates were separated by centrifugation, resulting in nonsoluble (ns) and soluble (s) fractions. The protein fractions obtained were electrophoresed by SDS−PAGE (9−15% polyacrylamide gradient). While radioactivity was detected autoradiographically (A), the proteins were stained with Coomassie (B). The immunostaining on Western blots (C) was carried out as given in the legend to Figure 6. The results of the 0.1% and 1% Triton X-100 treatment are shown with respect to the major phosphoproteins.

ecto-PK present in HeLa cells. For this, purified nucleolin and recombinant hnRNP U were reacted with intact HeLa cells in the presence of [γ-^{32}P]ATP under the selective conditions for detection of ecto-PK activity as detailed above, and as a control those reacted with recombinant CKII. Both proteins were found to be phosphorylated by ecto-PK and CKII activity. Autophosphorylation controls using hnRNP U, which since it is produced as a recombinant product offers the most stringent conditions for such a test, indicated that the protein was incapable of autophosphorylation (not shown). Purified nucleolin, however, indicated a minor degree of autophosphorylation (less than 5% as compared to phosphorylation by added PK) which most probably is due to an unavoidable contamination with CKII (F. Amalric, personal communication). With the phosphorylated proteins, phosphopeptide maps were generated as shown in Figure 8. Aside from one minor band (30 kDa) detectable only in the peptide map of authentic nucleolin, three comparable major

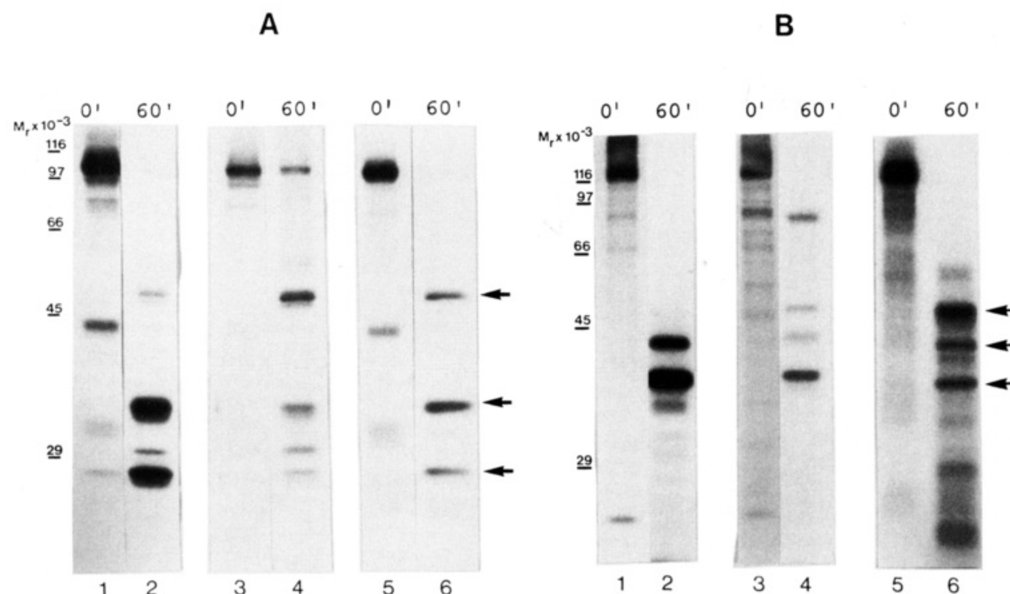

FIGURE 8: Comparative phosphopeptide mapping. Phosphorylation of 0.5 μg of purified nucleolin and hnRNP U was performed by using recombinant CKII or by the ecto-kinase activity as described under Experimental Procedures. For control, pp100 and pp120 were phosphorylated and isolated from a total of 10^7 HeLa S3 cells as described in Figure 4. Radioactive phosphopeptides were separated by SDS–PAGE in 12% polyacrylamide gels. Shown are the autoradiographic patterns after 0 and 60 min protease digest of (A) pp100 and nucleolin with V8 protease and (B) pp120 and hnRNP U with chymotrypsin. Lanes 1 and lanes 2, sample phosphorylation with CKII; lanes 3 and lanes 4, sample phosphorylation by ecto-PK; lanes 5 and lanes 6, isolated surface phosphoproteins. Arrows mark the position of the major phosphopeptides in each case.

phosphopeptides (50, 35, and 28 kDa) were observed in both pp100 and nucleolin. Likewise, pp120 and hnRNP U (phosphorylated by ecto-PK) cleavage also resulted in three identical phosphopeptides (46, 44, and 40 kDa) while the 44 kDa peptide was not generated by CKII-catalyzed hnRNP U phosphorylation. Despite the minor variations seen with both proteins, the results of the phosphopeptide maps suggest that similar sites were phosphorylated by ecto-PK in the surface phosphoproteins and their nuclear counterparts, although their different relative labeling intensities may relate to different preferences of phosphorylation sites.

DISCUSSION

This study is concerned with the isolation and identification of two major ecto-PK substrates present in many cell types, pp100 and pp120. As characterized here with HeLa cells, the phosphorylation of pp100 and pp120 in their extracellular domains occurring at Ser residues is sensitive to treatment of intact cells with low trypsin concentrations. The inability to cross-link pp100 and pp120 to each other or to other cell surface proteins and the results of comparative gel electrophoresis under reducing and nonreducing conditions indicate that both phosphoproteins occur in monomeric form. Purification and enrichment of pp100 and pp120 were achieved on the basis of radioactivity detection on and isolation from 1D- and 2D-gels as the method of choice. Our results show that pp100 is related to nucleolin, a major nucleolar RNA-binding protein (Lapeyre et al., 1987), and CKII substrate (Belenguer et al., 1990). The other phosphoprotein, pp120, proved to be affiliated with hnRNP U, which is known as 1 of the about 20 pre-mRNA-binding nucleoplasmic proteins (Kiledjian & Dreyfuss, 1992) and is also a phosphoprotein (Choi & Dreyfuss, 1984). The two ecto-phosphoproteins are indeed structurally related to known nuclear proteins, and therefore are referred to as nucleolin-like and hnRNP U-like, respectively, for the following independent reasons: (i) consensus amino acid sequences to the known nuclear protein sequences were found in several tryptic peptides; (ii) antibodies to nucleolin and hnRNP U cross-react with the ecto-PK substrates pp100 and pp120 as shown by colocalization of the immunostaining after Western blotting of 2D-gels and by immunoprecipitation of the ^{32}P-labeled surface proteins; (iii) proteolytic digestion of these two cell surface proteins and that of nuclear proteins phosphorylated by the ecto-PK of intact cells led to virtually the same phosphopeptide pattern.

The probability of sequences of this length occurring by chance ranges from $\sim 1 \times 10^{-4}$ to 1×10^{-17}; the probability of several within a given protein occurring coincidentally thus verges on the astronomical, and provides convincing evidence for a close relationship, if not identity, between the proteins. Additionally, the cross-reactivity of the antibodies implies the identity of the corresponding epitopes, typically six to eight residues in length. The presence of identical phosphopeptides in the tryptic digests serves to show that the consensus recognition sequences for the kinase are also homologous, making the chance that the protein pairs are not closely related vanishingly small.

Phosphorylation of pp100 and pp120 was obtained under stringent essential criteria for the specific detection of proteins phosphorylated on the surface of intact cells as detailed by us earlier (Kübler et al., 1982a,b, 1983; Kinzel et al., 1986): cell membrane barrier function, use of extracellular ATP at external sites, and demonstration of the surface location of the phosphorylation products as well as exclusion of material from dead or damaged cells. Important evidence for their surface location was deduced from treatment of intact cells with trypsin, presumed to limit its proteolytic activity to cell surface proteins. Cell trypsination removed the radioactive label of pp100 and pp120, while trypsination prior to ecto-PK phosphorylation prevented their labeling. Further support of the cell surface location came from the results of cell surface biotinylation showing

superimposition of labeled 2D-gel spots with ^{32}P-labeled pp100 and pp120. On the other hand, neither phosphoprotein was detectable on the surface of cells by routine immunofluorescence microscopy, most probably due to the presence of only about 10^4 copies per cell. Another common reason may be that antigenic sites of pp100 and pp120 are buried on the cell surface and are therefore not available for the antibodies used.

Although it is mostly believed that PK activities catalyze the transfer of phosphate from ATP to their substrates, it is important to note that a variety of phosphoaccepting proteins can undergo autophosphorylation and thereby can exhibit altered functions. Most PKs catalyze autophosphorylation [for a recent review, see Smith et al. (1993)], and also nucleotide (NTP)-binding proteins (Walker et al., 1982; Traut, 1994) including members of the GTP superfamily (Farnsworth & Feig, 1991; Newman & Magee, 1993), heat schock proteins (Csermely & Kahn, 1991; Huang et al. 1993), or immunoglobin-binding protein (Gaut & Hendershot, 1993) were shown autophosphorylated. It is of interest that hnRNP U, in contrast to nucleolin, carries a putative NTP-binding site (Kiledjian & Dreyfuss, 1992) and autophosphorylation may be a theme. However, using recombinant hnRNP U alone with [γ-^{32}P]ATP, we do not detect autophosphorylation, and it seems likely that a small amount of PK activity copurified with nucleolin (F. Amalric, personal communication) may mimic "autophosphorylation" of the nucleolin preparation. To assess the possibility of autophosphorylation of the surface substrates pp100 and pp120 directly, we have designed experiments which extinguish or reduce ecto-PK activity and thereby show that the detectable ^{32}P incorporation in pp100 and pp120 is a function of ecto-PK (CK) activity. First, under the influence of heparin as a specific inhibitor of protein kinase CK (Pyerin et al., 1987), surface protein phosphorylation was prevented while heparin treatment of surface-labeled cells was without effect on the phosphoproteins. Second, the phosvitin-induced shedding of ecto-PK shown earlier to occur in a selective manner (Kübler et al., 1983) was used to deplete cells from their ecto-PK. This treatment affects cell surface phosphorylation as indicated by drastically reduced labeling intensities while a preceding phosphorylation of cells, prior to ecto-PK release, did not bring about any change of radioactive labeling of pp100 and pp120. Thus, both types of data, besides giving evidence for the direct involvement of ecto-PK as a mediator of detectable pp100 and pp120 phosphorylation, also argue against detectable autophosphorylation. Whether other factors in the neighborhood such as from the extracellular matrix influence pp100 and pp120 phosphorylation needs further investigation.

In view of the occurrence of cell surface proteins which may be isoforms or genetic subtypes of nuclear proteins, a most interesting open question concerns the mechanism(s) for their cell surface expression. One obvious possibility for generation of the cell surface proteins would be a modification of the protein which leads to translocation signals and cell membrane insertion of the product. pp100 or pp120 association with the cell surface membrane, whether being peripheral or integral membrane proteins (Findlay, 1990), remains uncertain. While pp100 and pp120 were not dissociated from intact cells with isotonic buffers or from particulate fractions using salt concentrations in the range from 0.15 to 1.5 M NaCl, these phosphoproteins could be readily solubilized by cell extraction under low detergent conditions, suggesting a rather tight association with the cell surface membrane.

In the case of nucleolin, expression studies through Northern blots have revealed hybridizing signals which could indicate a mechanism of alternative pre-mRNA splicing (Srivastava et al., 1989). Another possibility relates to a putative second exon-1 in the genomic nucleolin sequence with predominantly hydrophobic amino acids (Srivastava et al., 1990) which could serve as a potential membrane anchor. A further important mechanism which eventually could lead to the proteins' cell surface location is by different polyadenylation sites in mRNA, as recently indicated for the case of hnRNP U (Fackelmayer & Richter, 1994; Fackelmayer et al., 1994).

The actual functions of proteins related to nuclear proteins at the cell surface have yet to be established. However, a 109 kDa LDL-binding protein occurs at the surface of HepG2 liver cells which was identified as nucleolin-related (Semenkovich et al., 1990), and a laminin-binding protein (LBP) having an 18 amino acid N terminus almost identical to nucleolin was purified from neuronal cells (Kleinman et al., 1991). In addition, there is some information on the occurrence of nucleolin serving as an autoantigen in systemic forms of autoimmune diseases (Minota et al., 1992; Wesierska-Gadek et al., 1992). Certainly, the CKII-like activity at the cell surface has a known nuclear counterpart, CKII.

To our knowledge, this is the first detailed biochemical report which demonstrates that proteins with nuclear protein-like properties occur among radioactively phosphorylated ecto-PK substrates. It should be noted that other highly phosphorylable nuclear proteins such as histones or cognate proteins such as hnRNP C1/C2 (Choi & Dreyfuss, 1984) did not appear within the spectrum of surface proteins. Further investigations at the mRNA and DNA levels will have to clarify the mechanism of the surface expression and the function(s) of pp100 and pp120 at the cell surface. Another important future question is whether there is communication between the peripheral and nuclear sites.

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