Ecto-protein kinase substrate p120 revealed as the cell-surface-expressed nucleolar phosphoprotein Nopp140: a candidate protein for extracellular Ca\(^{2+}\)-sensing

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A variety of cell membrane proteins become phosphorylated in their ecto-domains by cell-surface protein kinase (ecto-PK) activities, as detected in a broad spectrum of cell types. This study reports the isolation and identification of a frequent ecto-PK substrate, ecto-p120, using HeLa cells as a model. Data from MS and further biochemical and immunochemical means identified ecto-p120 as a cell-surface homologue of human nucleolar phosphoprotein p140 (hNopp140), which belongs to the family of argyrophilic (AgNOR-stainable) proteins. The superposition of \(^{32}\)P-labelled ecto-nucleolar phosphoprotein p140 (ecto-Nopp140) with anti-Nopp140 immunostaining could be demonstrated in a wide range of cell lines without any exceptions, suggesting a nearly universal occurrence of cell-surface Nopp140. A previous, tentative association of ecto-p120 with the nucleoplasmic pre-mRNA-binding protein hnRNP U has thus been supplanted, since improved purification techniques have allowed unambiguous identification of this ecto-PK cell-surface substrate. Furthermore, we have shown that rapid suppression of ecto-hNopp140 phosphorylation resulted upon a rise in the free extracellular calcium, while lowering the calcium concentrations returned ecto-Nopp140 phosphorylation to the original level. It is important to note that these Ca\(^{2+}\)-dependent effects on ecto-Nopp140 phosphorylation are not accompanied by alterations in the phosphorylation of other ecto-PK substrates. Our results indicate that, in addition to nucleolin, a further nucleolar protein, which was considered initially to be strictly intracellular, is identified as a cell-surface phosphoprotein.

Key words: ecto-enzyme, extracellular ion receptor, membrane protein, peptide analysis, phosphorylation.

INTRODUCTION

Cell-surface (ecto-) protein kinase (PK) activities enable cells to phosphorylate membrane-bound substrates and suitable extracellular molecules; these, in turn, have been shown to connect with important functions for the regulation of cellular development and cell–environment interactions [1]. Using intact cells for assaying ecto-PK activities [2–5], we found that a large range of proliferating and post-mitotic cell types carry serine/threonine ecto-PK with distinct substrate specificity, notably of the constitutively active CK2 (protein formerly known as casein kinase 2) and CK1 types [2,6], and cAMP-dependent PK [7].

Surface membrane-bound proteins that would represent physiological target proteins for CK2- and CK1-type ecto-PK [6] can be assessed by both conventional gel electrophoresis (SDS/PAGE) and autoradiography, in the latter case using lysates from surface \(^{32}\)P-labelled cells. Radioactive protein profiles of cells generally exhibited a large number of phosphoproteins (< 30), including some heavily labelled proteins that occurred with high frequency, such as the phosphoproteins p100 and p120 (named according their relative mobility in SDS/PAGE gels). Although easily detectable by radioactive labelling, when subjected to the (relatively insensitive) process of Coomassie Blue staining the ecto-proteins seem to occur in comparatively much smaller quantities, a phenomenon that has eluded efforts to determine their identity by biochemical characterization. Identification of the highly phosphorylated protein p100 (ecto-p100) was finally achieved by using a technique of radioactivity detection on and isolation from one-dimensional (1D) (SDS/PAGE) and two-dimensional (2D) (isoelectric focusing (IEF) + SDS/PAGE) gels. The analysis, including microsequencing of tryptic peptides, identified ecto-p100 unexpectedly as a homologue of nucleolin [8], which has a preponderant nucleolar association and is involved in ribosomal biogenesis and maturation [9–11]. Interestingly, cell-surface nucleolin has been reported by several other groups to act also as a cell-surface receptor, which, by shuttling between cytoplasm and nucleus, may additionally provide a mechanism for extracellular regulation of cell growth or virus infection [12–18].

A similar fundamental approach as for ecto-p100/nucleolin was used for enrichment and identification of ecto-p120, and the data initially indicated an affiliation with the nucleoplasmic pre-mRNA-binding protein hnRNP U [8]. In these experiments, it was repeatedly observed, however, that only a small portion of total ecto-p120 entered the IEF gel, and therefore a large number of 2D gel spots had to be eluted in order to obtain sufficient material for molecular studies. When we changed the isolation procedure so that all enrichment of ecto-p120 was before IEF, we observed for the first time a slight but significant separation of ecto-PK radioactivity from the hnRNP U immunostain (unpublished work). This discrepancy put the identity of ecto-p120 with hnRNP U in question, and demanded further investigation.

Abbreviations used: CK2, protein formerly known as casein kinase 2; 1D/2D, one-/two-dimensional; ECL, enhanced chemiluminescence; (ecto-)PK, (cell-surface) protein kinase, IEF, isoelectric focusing; MALDI-MS, matrix-assisted laser desorption/ionization MS; Nopp140, nucleolar phosphoprotein p140; NOR, nucleolus organizer region.

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The present study addresses more extensive characterization of the ecto-PK substrate p120 using techniques only made possible since optimization of the enrichment procedure. Specifically, we have used tryptic peptides of the isolated ecto-p120 for MS analyses, which indicate sequence homology with a nucleolar protein which belongs to the family of argyrophilic (AgNOR-stainable) proteins. By means of immunochromical techniques and further biochemical analyses, the presumed relationship has been confirmed and extended to different cell lines. Upon an elevation of free extracellular calcium, the ecto-p120 surface phosphorylation was abrogated, whereas lowering the calcium concentrations returned phosphorylation of this cell-surface protein to the original level. These results suggest that cellsurface phosphorylation of ecto-p120 may have a functional role in responding to extracellular calcium levels.

**EXPERIMENTAL**

Cell culture media and the serum supplements were obtained from Gibco BRL (Karlsruhe, Germany). Trypsin (from pig) for protein digests in gels was obtained from Promega (Mannheim, Germany) and the protease Asp-N from Boehringer-Roche (Mannheim, Germany). Amethopterin, adenosine, thymidine, ATP, 4-chloro-1-naphthol and 4,6-diamidino-2-phenylinodole were obtained from Sigma (Munich, Germany) and Benzonase, and the proteinase Asp-N from Boehringer-Roche (Mannheim, Germany). The material for enhanced chemiluminescence (ECL®) was from NEN Life Science Products (Zaventem, Belgium). All other reagents and chemicals were of the highest grade available. Bacterially expressed monomeric protein kinase CK2α and heteromeric CK2α/β were kindly provided by Dr W. Pyerin and Dr A. Krehan (DKFZ, Heidelberg, Germany).

**Antibodies and antisera**

Monoclonal antibody HC2 (raised against purified nucleolar protein p130) was generously given by Dr Ning-Hsing Yeh (University of Taipei, Taiwan, Republic of China). Polyclonal rabbit anti-Nopp140 serum was raised against the peptide sequence (residues 292–309) of Nopp140 and kindly provided by Dr U. T. Meier (Albert Einstein University, New York, NY, U.S.A.) and anti-(hnRNP U) (3G6) was from Dr S. Pin (University of Taipei, Taiwan, Republic of China). Polyclonal rabbit anti-Nopp140 serum raised against the peptide sequence (residues 221–232) coupled with keyhole-limpet haemocyanin was kindly provided by W. Pyerin and Dr A. Krehan (DKFZ). Anti-p120 proliferation-associated nucleolar antigen (AM-2385-11; mouse IgG1), raised against partially purified human nucleolar antigen, was given by Dr W. Pyerin and Dr A. Krehan (DKFZ). Anti-p120 proliferation-associated nucleolar antigen (AM-2385-11; mouse IgG1), raised against partially purified human nucleolar antigen, was bought from InnoGenex (San Ramon, CA, U.S.A.) and further biochemical analyses, the presumed relationship has been confirmed and extended to different cell lines. Upon an elevation of free extracellular calcium, the ecto-p120 surface phosphorylation was abrogated, whereas lowering the calcium concentrations returned phosphorylation of this cell-surface protein to the original level. These results suggest that cellsurface phosphorylation of ecto-p120 may have a functional role in responding to extracellular calcium levels.

**Cells**

HeLa (monolayer) cells were seeded in 7 or 20 cm² tissue culture plates and propagated in minimal essential medium with 10 % (v/v) bovine serum in an atmosphere containing 5 %, CO₂ to the densities given for specific experiments.

The HeLa-S3 (suspension) cell line was obtained from the National Center for Infection and Diseases, Atlanta, GA, U.S.A. through Dr E. Ades and Dr F. Candal (CDC) and Dr T. Lawley of Emory University. Other cell types employed for analysing cell-surface phosphoprotein spectra, as shown in Figure 1 and Table 2, were propagated in their appropriate media.

**Figure 1 Cell-surface protein phosphorylation by ecto-PK**

Cells of different origin were phosphorylated in the presence of 1μ[^32P]ATP for 15 min, as detailed in the Experimental section. 32P-labelled cells were lysed with SDS sample buffer, and total cell lysates (from 7 × 10⁶ cells) were separated by SDS/PAGE (7.5–15 % gradient gels). Radioactivity was detected by autoradiography. Shown are patterns of 32P-labelled cell-surface proteins. The molecular masses of marker proteins and the location of two highly labelled cell-surface proteins, marked as p120 and p100 (cell-surface nucleolin [8]) respectively, are indicated for comparison. It is noteworthy that the exposure time for HeLa-S3 (suspension) cells relative to HeLa (monolayer) cells was shorter because the level of surface phosphorylation exhibited by HeLa suspension cells was always much greater (at least by a factor of 5). Cell lines used: HEL, embryo lung (human); A431, vulva; BAβ, lymphoblastoid (human); MG 63, osteosarcoma (rat); RK 13, kidney (rabbit); NIH3T3 and SR 3T3, fibroblasts (mouse); C3H, liver (rat); PC 12, pheochromocytoma (rat); RSV-CEC, Rous sarcoma virus-transformed embryonic chick. (Chicken).

**Western blotting, AgNOR-staining and immunostaining**

Proteins separated by 1D- or 2D-electrophoresis were blotted on to Immobilon-P (PVDF) membranes using the semi-dry system.
described by Kyhse-Andersen [20]. Protein on Immobilon-P was stained with Ponceau S or Amido Black.

The specific staining of certain nucleolar proteins using a one-step silver-staining method with Gelatin Colloidal Developer (AgNOR-staining) was performed according to the protocol described by Hozak et al. [21].

For immunodetection, the Immobilon-P was blocked with PBS-Tween/3% (w/v) BSA, and then incubated with the primary antibodies for 2 h, as described for each particular experiment. Binding of primary antibodies was visualized by ECL* using, as horseradish-peroxidase-conjugated secondary antibodies, sheep anti-mouse or rabbit anti-human antibodies at a dilution of 1:2000. Alternatively, the immunoblots were stained with 0.03% (v/v) 4-chloronaphthol solution containing 50 mM Tris/HCl, pH 7.4, 0.03% (v/v) H$_2$O$_2$ and 0.001% (v/v) redox enhancer [p-phenylenediamine (100 mg/ml)/sodium metabisulphite (50 mg/ml)].

The antibody specificity and background staining were controlled by using cell lysate proteins that were separated by SDS/PAGE [10% (w/v) polyacrylamide gels], followed by Western blotting. Examples of specific staining are given in the Results section.

Immunoadsorption experiments

Cell lysates for the immunochemical experiments were prepared as described by Chen et al. [22]. The cells were suspended in 1.5 ml of buffer solution containing 50 mM Tris/HCl, pH 7.5, 1% (v/v) Triton X-100, 0.15 M sodium chloride, 2 mM dithiothreitol and one tablet of Mini Complete (EDTA-free; from Boehringer-Mannheim) and 50 ml of lysis buffer as the proteinase-inhibitor cocktail. The cell lysis solution was kept on a rotary shaker for 15 min at 37 °C. The cell supernatant fluid was recovered, and three subsequent cycles of washing with chilled assay buffer containing 1 mM unlabelled ATP. Washing cells were immediately lysed with sample buffer containing 2.5% (w/v) SDS and 2 μl of Benzonase was added to 20 μl of the lystate. For incorporation of the radioactivity into proteins, the samples were subjected to 1D or 2D gel electrophoresis, as described below.

Protein phosphorylation on immunobeads was accomplished with beads suspended in 100 μl of phosphorylation assay buffer in the presence of recombinant protein kinase CK2α. The reaction was started by the addition of 2 μl of protein kinase CK2α (3 μg/μl) and [γ-32P]ATP (final concentration 2 μM), and allowed to proceed for 15 min at 37 °C. The reaction was terminated by removal of beads from the supernatant fluid, and three subsequent cycles of washing with chilled assay buffer containing 1 mM unlabelled ATP (see above) and centrifugation at 12000 g for 5 min. SDS sample buffer (60 μl per 50 μl beads) was added and the slurry was heated up to 95 °C for 5 min. Radiolabelled proteins on gels or Western blots were detected by autoradiography, as described below.

Phosphopeptide mapping

Radiolabelled ecto-hNopp140 was obtained after cell-surface phosphorylation (10^6 cells) (or labelled hNopp140 was obtained after adsorption to antibody [HC2 or anti-(Nopp140)]-derivatized immunobeads (using lysate from 2 × 10^6 cells)) and reaction with protein kinase CK2α (as described above). The samples were electrophoresed by SDS/PAGE (6% gels) and examined for radiolabelling. The corresponding Nopp140 gel bands were cut out, washed three times with distilled water, then washed twice with a mixture of acetonitrile/water (1:1, v/v), and then once with acetonitrile. This treatment caused shrinking of the gel pieces, which were then re-swollen with 100 μl of 50 mM Tris/HCl, pH 7.4, containing 8 μl of the proteinase Asp-N (Boehringer Mannheim) at room temperature for 3 h, followed by incubation at 37 °C for 15 h. The cleavage was stopped by the addition of 100 μl of SDS sample buffer and heating the sample to 95 °C for 15 min. The radioactively labelled fragments were separated by SDS/PAGE (12% gels).

Gel electrophoresis and detection of radiolabelled proteins

1D separation of proteins was achieved by SDS/PAGE; 2D separation was achieved by IEF (pH range 4–7) in the first dimension and SDS/PAGE in the second dimension, as described for specific experiments. Radiolabelled phosphoproteins were visualized by autoradiography (X-Omat; Kodak, Rochester, NY, U.S.A.) or phosphorimaging (PhosphoImager; Fuji, Straubinghardt, Germany).

Matrix-assisted laser desorption/ionization MS (MALDI-MS)

This analysis was performed by Dr T. Kempf and Dr M. Schnölzer from the ‘Zentrale Proteinanalytik’ group of the DKFZ. Proteins on 2D gels localized by autoradiography were
excised from the gel, repeatedly washed with water and water/acetonitrile, and digested overnight with trypsin solution (0.4 µg of trypsin/30 µl of 50 mM ammonium hydrogen carbonate) at 37 °C. The generated peptides in the supernatant were analysed by MALDI-MS, as described in [24]. Briefly, aliquots of 0.3 µl of a nitrocellulose-containing saturated solution of α-cyano-4-hydroxycinnamic acid in acetone were deposited on to individual spots on the target. Subsequently, 0.8 µl of 10% (v/v) formic acid and 0.4 µl of the digest sample were loaded on top of the thin film spots, and allowed to dry slowly at ambient temperature. To remove salts from the digestion buffer, the spots were washed with 10% (v/v) formic acid and with water. MALDI mass spectra were recorded in the positive-ion mode with delayed extraction on a Reflex II time-of-flight instrument (Bruker-Daltonik GmbH, Bremen, Germany) equipped with a SCOUT multiprobe inlet and a 337 nm nitrogen laser. Ion acceleration voltage was set to 20.0 kV; the reflector voltage was set to 21.5 kV, and the first extraction plate was set to 15.4 kV. Mass spectra were obtained by averaging 50–200 individual laser shots. Calibration of the spectra was performed internally by a two-point linear fit using the autolysis products of trypsin at m/z 842.50 and m/z 2211.10. Peptides were identified by computer-assisted analysis using the peptide mass database in the program ProFound.

RESULTS

Detection and identification of the cell-surface phosphoprotein ecto-p120

Ecto-protein kinase-mediated phosphorylation reveals isoforms of ecto-p120

The phosphorylation of cell-surface proteins as a result of ecto-PK activities using exogenous [γ-32P]ATP (or [γ-32P]GTP) as the 32P donor yields the highly labelled surface protein ecto-p120 among the cell-type-specific phosphoproteins, as illustrated for several cultured cell types in Figure 1. The percentage of radioactivity incorporation into ecto-p120 in, for example, HeLa cells ranges from 5–12% of total surface 32P-labelling, as determined from relative phosphorylation intensities in the PhosphoImager measurements. When separated by IEF in the first dimension and SDS/PAGE (8% acrylamide) in the second dimension, the ecto-p120 radioactivity was resolved as three or four distinctive spots in the range of pH 5.3–5.7, indicating isoelectric variants (shown for HeLa cells in Figure 2). When

![Figure 2](image-url)  
**Figure 2** 32P-labelled ecto-proteins resolved in two dimensions by IEF and SDS/PAGE  
Phosphorylated HeLa cells were lysed and material equivalent to ~5 × 10⁶ cells was subjected to IEF in the pH range of 4–7, followed by SDS/PAGE (8% gels) as detailed in the Experimental section. Shown is a typical 2D pattern of radioactively labelled phosphoproteins by autoradiography. Marker proteins and major phosphorylated surface proteins, p120 and p100, are indicated. The inset shows p120 radioactivity, as obtained in 2D gels using 6% gels for separation in the second dimension.

![Figure 3](image-url)  
**Figure 3** Ecto-p120 radiolabelling correlates with argyrophilic protein silver-staining (NOR)  
Material equivalent to 5 × 10⁶ cells was loaded for 2D gel electrophoresis (8% gel for SDS/PAGE in the second dimension). Western blotting to Immobilon-P, radioactivity detection by autoradiography and NOR staining were as described in the Experimental section. The results show those parts of the 2D blot relevant for comparison with the major cell-surface phosphoproteins. It should be mentioned that, besides the strong NOR-stain of the two components shown here, a few other, albeit weakly stained, components were detectable in other areas of the blot. Identical results showing a positive correlation of NOR staining with radiolabelled ecto-p120 were obtained with different cell types.
lower acrylamide concentrations (6%) were employed for separation in the second dimension, the ecto-p120 isoforms could be resolved further as doublets with slightly different gel-migration rates (Figure 2, inset), suggesting the existence of two principal forms of ecto-p120.

Rates of $^{32}$P incorporation into ecto-p120 were determined by PhosphoImager analysis, averaged as 0.1 pmol of phosphate/10^6 HeLa cells measured against radioactivity standards. On the other hand, diminishingly small amounts of p120 protein coincided with the radioactivity (results not shown), and estimations of the abundance of p120 protein from protein-stained gels yielded values of $\approx$ 1 pmol/10^6 cells. Both on the basis of this default value and the observation that ecto-p120 is presumably a minor component (assumed to be $< 10\%$) relative to the total amount of protein in radioactive p120 (see Figure 4 below), the molar level of ecto-p120 labelling was estimated to be in the range of at least 1 mol of phosphate/mol of ecto-protein.

Western blot analyses (Figure 3) demonstrated that ecto-p120 radioactivity coincides with a specific silver-staining protein, which is characteristic of a certain set of argyrophilic proteins of the nucleolar organizer region (NOR). A positive correlation is likewise revealed for cell-surface nucleolin (ecto-p100), as might be expected since nucleolin is a prototypical NOR-stainable protein. These relationships suggested that ecto-p120 might be a member of the AgNOR protein family.

Table 1 Peptide masses of tryptic fragments from ecto-p120 and relationship to the human nucleolar phosphoprotein Nopp140

<table>
<thead>
<tr>
<th>Measured mass (M–H$^+$)</th>
<th>Computed mass (M–H$^+$)</th>
<th>Peptide sequence</th>
<th>Residue position in …</th>
<th>Missed cut</th>
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<tbody>
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<td>736.41</td>
<td>736.43</td>
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<td>hNopp140x</td>
<td>198–204</td>
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<tr>
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<td>1360.66</td>
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<td>hNopp140y</td>
<td>628–638</td>
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<tr>
<td>1688.02</td>
<td>1687.97</td>
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<td>hNopp140y</td>
<td>9–23</td>
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<tr>
<td>1816.96</td>
<td>1816.95</td>
<td>NKPGYSSVPPSAPPK</td>
<td>hNopp140y</td>
<td>292–309</td>
</tr>
<tr>
<td>1945.05</td>
<td>1945.04</td>
<td>NKPGYSSVPPSAPPK</td>
<td>hNopp140y</td>
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Isolation of ecto-p120 and its identification as a nucleolar phosphoprotein

Owing to the minute amounts of ecto-p120 protein, detection of the incorporated radioactive label was required in order to follow its isolation. In order to obtain the protein quantities sufficient for the subsequent microanalyses, we started with bulk quantities of surface $^{32}$P-labelled HeLa suspension (HeLa-S3) cells. Cells were lysed with buffer containing 1% (w/v) SDS, and the lysate was subjected to precipitation with chloroform/methanol, as described in the Experimental section. The resulting pellet was resolubilized in buffer for IEF. With starting material from $5 \times 10^7$ cells, we obtained approx. $3 \mu g$ of ecto-p120, as estimated from Coomassie Blue staining of 2D spots. In order to characterize radiolabelled ecto-p120 spots, the excised gel was digested with trypsin for MALDI-MS analysis. With the MALDI fingerprints, a search was executed in the peptide mass database included in the program ProFound (see the Experimental section). As shown in Table 1, the masses of seven tryptic peptides derived from ecto-p120 matched with those of the human nucleolar phosphoprotein p130, recently renamed as human Nopp140 (hNopp140) [22]. Two highly homologous forms of hNopp140, x and y, which essentially differ from each other by an insert of ten amino acid residues in y, exhibit molecular masses of 73720 Da and 74466 Da respectively, as deduced from their cDNA sequences [25,26]. Six out of the seven tryptic peptides obtained from the ecto-p120 were compatible with both hNopp140 isoforms. However, one single peptide (NKPGYSSVPPSAPPKK) matched up with hNopp140y specifically, because of a tandem exchange of two amino acids [shown underlined in the preceding sequence: SV (Ser-Val) in y, instead of YA (Tyr-Ala) in Nopp140x]. These data demonstrate that hNopp140y is a component of the radioactive ecto-p120. On the other hand, it is reasonable to assume that hNopp140x is present as well, since two radioactively labelled ecto-p120 bands, which would correspond to the slightly different molecular masses of Nopp140x and y, were distinguished in gel electrophoresis (see Figure 2 above).

Immunological confirmation for ecto-p120 relation to hNopp140

The data from MS (Table 1) were in complete agreement with predicted fragmentation patterns from the hNopp140 protein forms. Therefore we performed Western immunoblotting and immunoadsorption experiments by using monoclonal (HC2) as well as polyclonal (anti-Nopp140) antibodies. Western immunoblot data of 1D and 2D gels confirmed that the ecto-p120 radioactivity indeed corresponded with immunostaining by both anti-Nopp140 antibodies (Figure 4A). It should be noted here that the immunostaining of the ecto-Nopp140 protein would be expected to be minor, relative to the higher proportion of intracellular Nopp140. In contrast with anti-Nopp140 staining, no correlation of ecto-p120 radioactivity with immunostaining occurred when using antibodies against hnRNP U (a 120 kDa nucleoplasmic phosphoprotein, which was previously assumed to be affiliated with ecto-p120 [8]) or antibodies against the p120 human proliferation-associated nucleolar antigen [27] (results not shown).

The detection of an immunoreactive band with anti-Nopp140 antibodies at the same position in SDS/PAGE as the radioactive ecto-p120 protein indicated either cross-reactivity of the antibody or cross-contamination of the proteins. Thus immunoadsorption with Nopp140-derivatized Protein-A beads was performed from cell lysates of surface $^{32}$P-phosphorylated cells, followed by elution and Western immunostaining with anti-Nopp140 anti-
Figure 4  Ecto-p120 cross reacts with anti-Nopp140 antibodies

(A) Correlation of immunostaining by anti-Nopp140 antibodies with 32P labelling. Radiolabelling of HeLa-S3 surface proteins and separation by 2D gel electrophoresis (material equivalent to 4 x 10^6 cells was loaded) were as described in the legend to Figure 2. Western immunostaining and detection by ECL was as described in the Experimental section. Very similar immunostaining was obtained by using either monoclonal mouse anti-(hNopp140/p130) antibody (HC2; [25]) or polyclonal rabbit anti-Nopp140 antibody [27]. Shown is a typical result of immunostaining with antibody HC2 (upper panel) and correspondent ecto-p120 radioactivity (lower panel). (B) Immunoadsorption of ecto-p120 from cell lysates. The production of the starting cell lysates and the generation of antibody (HC2)-conjugated Protein A beads was performed and analysed as described in the Experimental section. Shown (in lanes from left to right) is: (a) 32P labelling of the starting cell lysate (from 0.7 x 10^6 surface-labelled cells) after SDS/PAGE (8% gels)/autoradiography; (b) 32P label after immunoadsorption to HC2 beads (starting material from 9 x 10^6 cells); and (c) 32P label of material after immunoadsorption of non-radioactive cell lysate (from 9 x 10^6 cells) and subsequent phosphorylation of the bead-bound material (as detailed in the Experimental section). For comparison with lanes (a) and (b), the autoradiographic exposure time of lane (c) was approx. 7 times shorter, due to significantly higher radiolabelling. The adjacent lane right of lane (c) shows Western-blot immunostaining of lane (b) using antibody HC2 for positive control of Nopp140 immunoadsorption.

Whether ecto-p120/hNopp140 protein differs from intracellular Nopp140 is yet unclear. To explore further the degree of homology between these protein forms, we traced their 32P-phosphorylation by phosphopeptide mapping. Radiolabelled ecto-p120/hNopp140 was obtained after selective ecto-PK-mediated surface phosphorylation, while labelled intracellular Nopp140 was obtained by Nopp140 immunoadsorption of non-radioactive cell lysates (i.e. without previous radioactive surface phosphorylation), followed by radioactive phosphorylation of the loaded immuno-beads in vitro with CK2 activity as the ecto-PK-like catalysing enzyme. As might have been expected in view of its nature as an excellent CK2 substrate [28], we found strong 32P labelling of the bead-bound protein, which principally consisted of intracellular Nopp140 (Figure 4B). Its phosphopeptide map obtained after protease Asp-N digestion, when compared with that of ecto-Nopp140, revealed several matching peptides in the size range of 33–96 kDa, including a further major peptide at approx. 22 kDa (Figure 5). These data, demonstrating very similar or identical radioactive phosphorylation sites, concomitantly support the close correlation between ecto-p120 and hNopp140.

Effects of extracellular calcium on surface phosphorylation of ecto-hNopp140

Our previous studies have provided evidence that the ecto-PK-mediated cell-surface protein phosphorylation depends on magnesium, consistent with the formation of the enzyme co-substrate complex MgATP2−. It is striking, however, that the radioactive phosphorylation of ecto-Nopp140 was impaired in the presence of free extracellular Ca2+ ions, while the effect on radiolabelling of other ecto-substrates remained largely unchanged, as shown by the associated concentration-dependent regression of radioactivity incorporation (Figure 6). Similarly, markedly different autoradiographic signals were obtained by sample analysis after the preparation of the 2D gels. In addition, these targeted calcium effects were detected in cells grown to different final cell densities known to carry different levels of ecto-PK activity [3] (Figure 6). Taken together, these results suggest that it is mostly probable the ecto-Nopp140 protein itself that responds to Ca2+ ions in some way, rather than the catalysing enzyme. In addition, treatment of surface-phosphorylated cells with Ca2+-containing buffer did not significantly compromise ecto-Nopp140 phos-
Ecto-p120 revealed as cell-surface Nopp140

Figure 6 Ecto-hNopp140 phosphorylation: influence of extracellular calcium

Surface protein phosphorylation of HeLa cells was performed under standard conditions as in Figure 1, but in the absence (−) or presence (+) of Ca²⁺, as indicated. Identical aliquots of radioactive cell lysates (equivalent to 5 × 10⁵ cells) were separated by SDS/PAGE (8% gels), and radioactivity was detected by autoradiography. (a) Shown are typical results from radiolabelling cell-surface proteins from the same set of cells (cell density of 1.7 × 10⁶/cm²). The specifically altered radioactivity of p120/ecto-Nopp140 should be noted. (b) Dose–response experiments for Ca²⁺ at the indicated concentrations (cells as in panel a). Parts of the autoradiographic results relevant for p120/ecto-Nopp140 radioactive phosphorylation are reported. (c) Response to 2 mM Ca²⁺ using cell cultures of different cell growth density, as indicated. The relevant section of p120/ecto-Nopp140 radiolabelling is shown.

Table 2 Incidence of the ecto-hNopp140 (formerly known as ecto-p120) in a selection of different cell types

<table>
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<th>Cell type</th>
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Table 2 shows the incidence of the ecto-hNopp140 (formerly known as ecto-p120) in a selection of different cell types. Cell-surface phosphorylation with intact cells and autoradiographic detection of ³²P-labelled proteins after 1- or 2D-acrylamide gel electrophoresis was performed as described in the legends to Figures 1 and 2. Immunostaining of Western blots with specific anti-Nopp140 antibodies was as given in the legend to Figure 4. The analogy of ecto-p120 phosphorylation and positive antibody staining was evaluated on a strictly qualitative basis using ‘+’ when detected; ‘(+)’ indicates a general low level of surface phosphorylation.

The possibility was considered that changes in ecto-Nopp140 phosphorylation might result from a potentially reversible Ca²⁺-dependent substrate modulation. Although the molecular details of such a mechanism provide a compelling question for future studies, in principle one should expect reversible changes in ecto-Nopp140 phosphorylation, which would occur rapidly after setting the specific conditions. One favoured approach implied sequestration of extracellular Ca²⁺ and restoration of the ecto-Nopp140 phosphorylation in the presence of a chelating substance, such as EDTA or EGTA. It became evident (Figure 7) that the abrogation of ecto-Nopp140 phosphorylation seen at 2 mM Ca²⁺ could not only be immediately reversed in the presence of EGTA and, with less sensitivity, by EDTA, but the chelators led to ³²P phosphorylation intensities that exceeded those of the controls in the absence of Ca²⁺. Moreover, increased levels of ecto-Nopp140 phosphorylation occurred in the presence of 1–2 mM EGTA alone (Figure 7). Whether this result might imply that threshold Ca²⁺ ions normally associated with ecto-Nopp140 attenuate the degree of ecto-Nopp140 phosphorylation remains to be established.

Distribution of ecto-hNopp140

As demonstrated in Figure 1, several cell lines of different origin and differentiation state showed the intensely phosphorylated protein ecto-p120 among the membrane-bound ecto-PK substrates. After ecto-p120 was identified as ecto-hNopp140 in HeLa cells, we extended the study to explore whether this correspondence is also valid for other cell types, and we questioned whether phosphorylated ecto-p120 gel bands correspond to anti-Nopp140 antibody staining similar to that shown in Figure 4(A) above. The results obtained with several cell types are summarized in Table 2. It is evident that ecto-p120 phosphorylation unambiguously coincided with the immunostaining by anti-Nopp140 antibodies in all cells employed, pointing to...
very similar, or identical, epitopes of the Nopp140 proteins from different cell species.

**DISCUSSION**

In our attempt to identify the prevalent ecto-PK substrate p120, we have disclosed the homology of this protein with the human non-ribosomal nucleolar phosphoprotein Nopp140 (hNopp140). hNopp140, a 130 kDa protein [25,26], is an essential constituent both of the fibrillar components of nucleoli and of coiled bodies (outside of the nucleolus), which are both AgNOR-stained structures [29,30]. Our data (Figure 3) showing coincidence of AgNOR staining after Western blotting with radioactive ecto-hNopp140 (p120) and ecto-nucleolin (p100) agree with their assignment as NOR proteins. Since different cell types showed the same overlap of 32P-labelled ecto-p120 with silver stain and anti-Nopp140 immunostain, ecto-hNopp140 appears to be a general constituent of the cell surface.

The expression of hNopp140 starting from two different RNA transcripts occurs in its isoforms a and b, which differ essentially by an insert of 10 amino acid residues [26]. Our MS data and the comparative search in a non-redundant protein database revealed that all but one peptide mass was included in both hNopp140 (a/b) forms; conversely, one peptide mass matched with a Nopp140b-derived peptide only (Table 1). This relationship directly demonstrated the presence of hNopp140b, but this does not exclude the presence of hNopp140a. In fact, two phenomena argue for the presence of both hNopp140 isoforms within the cell-surface hNopp140 radioactivity. One is that we obtained the ecto-protein as two closely migrating forms upon separation in low-percentage polyacrylamide SDS-gels (Figure 2). The other is that co-expression studies in different cell lines suggest that the a-form always exceeds the concentration of the b-form by a factor of 4–5 [26].

Further evidence for the close correspondence of ecto-p120 and hNopp140 was derived from immunochemical experiments with Nopp140-specific antibodies. Although Western blots showed that the areas of ecto-p120 radioactivity completely corresponded with anti-Nopp140 staining, this left open the possibility of cross-contamination of the two proteins. In contrast, the immunoadsorption assays with anti-Nopp140-derivated Protein A beads demonstrated cross-reactivity with radiolabelled ecto-p120. In view of the rich source of potential substrates present in total cell lysates, this result underlines the selectivity of the immunoassay employed here. In addition, the comparison of proteolytic cleavage products obtained from surface-labelled ecto-p120 and Nopp140 phosphorylated by addition of CK2 to immunobeads generated identical phosphopeptides, which can be expected only from molecules with very similar or identical structures. In this context, it should be pointed out that Anderer and colleagues [31,32] found indirect evidence for a Nopp140-like protein on the cell surface, in addition to its nucleolar location, but dismissed this as an artefact due to incompletely controlled experimental conditions.

Selective phosphorylation by ecto-PK appears appropriate for mapping cell-surface phosphoproteins in different cell types, and thereby contributes to the general knowledge of cell-surface molecules. The present study shows that cell-surface phosphorylation is a suitable means of discriminating ecto-hNopp140, in contrast with immunostaining, which reacts with all Nopp140 protein. A rough approximation of the labelling stoichiometry on the basis of 32P incorporation rates and protein amounts led to the estimate that at least 1 mol of phosphate per mol of ecto-Nopp140 is transferred by cell-surface phosphorylation. On the other hand, Nopp140 protein was found to be phosphorylated to unusually high degrees, which is not unexpected since it contains 82 serine residues in acidic serine repeats, of which over half occur in consensus CK2 phosphorylation sites [33]; indeed, levels corresponding to 75 phosphoryl groups per molecule have been reported [34]. As shown by gel-migration assays, the unphosphorylated precursor protein (100 kDa), upon massive phosphorylation, undergoes a typical conversion into Nopp140 [33]. Therefore the relatively low molar degree of actual surface labelling of ecto-Nopp140 and its co-migration with intracellular Nopp140 might indicate that either the majority of acceptor sites already existed in a phosphorylated state, or they were simply not available for the ecto-PK reaction.

The strong and selective depression of ecto-Nopp140 phosphorylation in response to the presence of external Ca2+ ions (Figures 6 and 7) appears to be important, since it was suggested that extracellular Ca2+ serves as a distinct form of intercellular signalling [35]. The concentration of extracellular Ca2+ in mammalian blood is kept near-constant at ≈ 1–1.3 mM through Ca2+-sensing and Ca2+-mobilization into and out of the extracellular fluid [36]. It is striking that the relative Ca2+ levels are monitored and signalled in certain tissues by extracellular Ca2+-sensing receptors, the activity of which is regulated at millimolar concentrations of the physiological agonist [37]. Given this, the demonstration of extracellular Ca2+ effects on ecto-Nopp140 phosphorylation in the concentration range approximating to that found physiologically seems an important feature, which fosters the idea that cell-surface Nopp140, through variable phosphorylation, may be involved in extracellular Ca2+-sensing.

In this report, I have demonstrated for the first time that the cell-surface PK-substrate ecto-p120 present in a wide range of cell types is a homologue of the nucleolar phosphoprotein Nopp140, a member of the family of argyrophilic (AgNOR-stainable) proteins. This is the second example of a nucleolar protein expressed at the cell surface. Ecto-nucleolin (p100), the first example, has been accepted to exist in this manner, since it has been shown to fulfil a variety of functions as a cell-surface receptor for different ligands, including viral proteins. One might speculate that ecto-Nopp140 could have similar functions. The idea that ecto-PK-mediated phosphorylation of ecto-Nopp140 via its modulation by extracellular calcium might contribute to cellular phenotype or function by sensing extracellular Ca2+ levels is an exciting possibility, which merits further investigation.

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**REFERENCES**


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