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PHOSPHORYLATION OF THE HUMAN CELL PROLIFERATION-ASSOCIATED NUCLEOLAR PROTEIN P120

Benigno C. Valdez, Rose K. Busch, and Harris Busch

Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030

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The human cell proliferation-associated nucleolar protein p120 was found in a variety of human cancer specimens but not in most normal resting cells. Polyclonal antibodies raised against bacterially expressed p120 were used to immunoprecipitate the p120 protein isolated from ^{32}P-labeled HeLa cells. The p120 protein was phosphorylated at serine, threonine and tyrosine residues. A tryptic peptide map showed it contained three labeled peptides. One of these peptides comigrated with a p120 peptide phosphorylated in vitro by casein kinase II. This peptide was phosphorylated in vitro both at Ser-181 and Thr-185. This region is juxtaposed to the epitope site recognized by the anti-p120 monoclonal antibody.

The p120 protein is a human cell proliferation-associated nucleolar antigen with an apparent molecular weight of 120,000. This antigen was detected in a variety of malignant tumors but not in most normal resting cells (1). This protein appears in the early G1 phase of the cell cycle (1, 2) and was observed in a network of beaded microfibrils in the nucleolus (3). The importance of p120 to cell proliferation was shown by the inhibition of cell growth and DNA and RNA syntheses when anti-p120 monoclonal antibody was microinjected into HeLa cells (4). The epitope region recognized by this anti-p120 monoclonal antibody has the sequence EAAAGIQW located at amino acid residues 173-180 (5).

Since p120 is associated with cell proliferation, we have studied its phosphorylation as part of our efforts to characterize its structure and function. This was made possible by the

Abbreviations: p120, protein with apparent molecular weight of 120,000; nt, nucleotide(s); bp, base pair(s); SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; NEM, N-ethylmaleimide; MW, molecular weight; MBP, maltose binding protein; MBP120.1 and MBP120.21, maltose binding protein with p120 peptides.

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production of anti-p120 polyclonal antibodies raised against p120 expressed in E. coli. These antibodies were then used for immunoprecipitation of in vivo phosphorylated p120. One of the three tryptic peptides of the in vivo phosphorylated p120 comigrated with a p120 peptide phosphorylated in vitro by casein kinase II at Ser-181 and Thr-185.

MATERIALS AND METHODS

Cloning and expression of p120 cDNA. The full-length p120 cDNA was constructed from the longest partial p120 cDNA isolated from a fetal liver cDNA library (6) and from genomic fragments (7). The fetal liver p120 cDNA was digested with EcoRI, and the shorter fragment (cDNA/SF in Figure 1) was isolated and partially digested with PstI. This fragment was subcloned into the PstI-

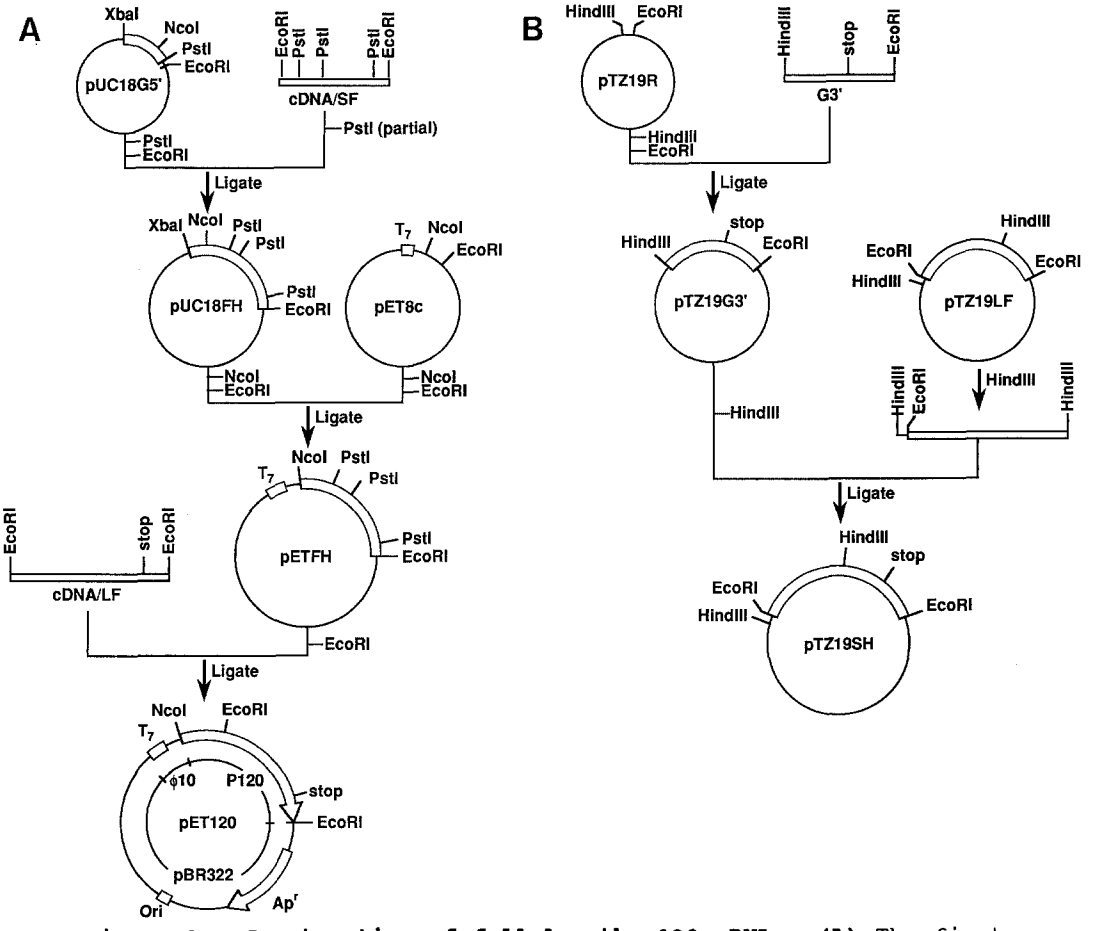


Figure 1. **Construction of full-length p120 cDNA.** **(A)** The first half was constructed using the 5' genomic clone (pUC18G5') and PstI-partially digested EcoRI fragment of the cDNA (cDNA/SF). **(B)** The second half was constructed using the 3' end of genomic clone (G3') and EcoRI fragment of the cDNA (insert in pTZ19LF). pTZ19SH was digested with EcoRI and the insert was subcloned in pETFH.

EcoRI sites of pUC18G5' which contained an XbaI-PstI genomic fragment (nt -280 to 103), resulting in clone pUC18FH. The NcoI-EcoRI fragment (nt 1 - 744 of p120 cDNA) of pUC18FH was subcloned into pET8c (8). The 3' half of the cDNA was constructed using two clones, pTZ19LF which contained the longer EcoRI fragment of the fetal liver cDNA and pTZ19G3' which contained the HindIII-EcoRI fragment at the 3' end of the genomic clone (Figure 1B). The resulting clone, pTZ19SH, contained the p120 coding region from nt 745 through the stop codon and an additional 400-bp 3' untranslated region. The insert in pTZ19SH was subcloned at the EcoRI site of pETFH joining the two cDNA fragments. The resulting clone, pET120, contained a full-length p120 cDNA sequence. The insert was expressed using E. coli strain BL26(DE3)pLysE according to Studier et al. (8).

Production of anti-p120 polyclonal antibodies. Electrophoresis of E. coli extract containing expressed p120 protein was done on a preparative SDS-polyacrylamide (8%) gel and a region corresponding to a molecular weight of 120,000 was excised. Protein p120 was electroeluted (9) from these gel slices, emulsified initially with Freund's complete adjuvant, and injected subcutaneously into two rabbits. Incomplete adjuvant was used for subsequent injections which were given intradermally, subcutaneously and intramuscularly. Approximately 100-400 μg of protein were used per injection for a total of three injections per rabbit at 3-4 weekly intervals. The animals were boosted and bled at monthly interval. A protein A affinity column (Pierce) was used to convert the rabbit serum to IgG. The sera were assayed by the ELISA procedure (10), Western blot analysis (Promega) and indirect immunofluorescence (1).

Radiolabeling and immunoprecipitation. HeLa cells in a 70-80% confluent 150 cm^2 flask was incubated in a phosphate- or methionine-free minimal essential medium with 5% fetal bovine serum (GIBCO) for 8 hours. The cells were then grown in a similar fresh medium containing either 0.8 - 1.0 mCi/ml [^{32}P]-orthophosphate (carrier-free) or 0.07 mCi/ml [^{35}S]-methionine (ICN) for 16-18 hrs. Nuclei were isolated as described previously (1) and lysed in triple-detergent lysis buffer according to Sambrook et al. (11). To minimize nonspecific immunocomplexes, 5 μl preimmune rabbit IgG and 100 μl ImmuBind Absorbent (GENEX) were added to 500 μl lysate, tumbled at 4^{o}C for 1 hr, and centrifuged. To the pretreated lysate, 10 μl rabbit immune IgG and 100 μl ImmuBind Absorbent were added. The slurry was tumbled at 4^{o}C for 1 hr and the immunocomplexes were separated, washed 4 times with RIPA buffer (11), and then with 10 mM Tris-Cl (pH 7.5), 0.1% NP-40, 1 mM PMSF, 2 mM NEM. Pellets were resuspended in 50 μl 2X Laemmli buffer containing 10% mercaptoethanol, boiled for 5 min and loaded onto SDS-polyacrylamide (8%) gel.

Cloning, expression and purification of maltose binding protein containing p120 peptides. Fragments of p120 cDNA were inserted at the BamHI site of pMalE178 (12). One fragment was a double strand synthetic oligonucleotide corresponding to nt 505 to 555 of p120 cDNA with GATC 5' overhung in both ends. A second longer fragment was produced by the polymerase chain reaction system. Amplification of nt 472 to 591 of p120 cDNA was done using synthetic oligonucleotide primers with BglII site. Maltose binding protein with or without p120 peptide was expressed in E. coli strain JM109 as described (12) and purified in one step by affinity chromatography. Bacterial pellets were suspended in Buffer A (10 mM Tris-Cl (pH 7.2), 0.02% NaN_3, 1 PMSF, 2 mM NEM), sonicated and centrifuged. The clear lysate was loaded onto a cross-linked amylose column (13) and washed with Buffer A. Bound

protein was eluted with 10 mM maltose in Buffer A and then concentrated using Centricon-30 (Amicon).

In vitro labeling, peptide mapping and phosphoamino acid analysis. The casein kinase II was a gift of Drs. E. Cardellini and E. Durban, Department of Pharmacology, Baylor College of Medicine, Houston, TX, purified from HeLa nuclei (14). Kinase reactions were carried out in 60 ul of 50 mM Tris-Cl (pH 8.0), 50 mM NaCl, 10 mM $MgCl_2$, 80 μCi of [γ-^{32}P]ATP (ICN), 1-2 μg of substrate and 10 ng of casein kinase II at 37^{o}C for 30 min. Reaction was stopped by the addition of an equal volume of 2X Laemmli buffer and then boiled for 5 min. Peptide mapping and phosphoamino acid analysis were performed as described (15, 16).

RESULTS

The bacterially expressed protein from pET120 plasmid immunoreacted with the anti-p120 monoclonal antibody and had the same migration on SDS-polyacrylamide gel as the p120 protein from HeLa cell nucleoli (data not shown). The patterns of partial digestion using N-chlorosuccinimide/urea for the two proteins were essentially the same as shown by migration of the immunoreactive peptides on SDS-polyacrylamide gel (6, 17). The polyclonal antibodies raised against *E. coli*-expressed p120 also immunoreacted with the p120 isolated from HeLa cell nucleoli. These results provide evidence that the p120 expressed from the cDNA clone is essentially the same as the p120 protein isolated from HeLa cell nucleoli.

The polyclonal antibodies against the bacterially expressed p120 protein immunoprecipited phosphorylated p120 protein. Its mobility on one-dimensional SDS-polyacrylamide gel was indistinguishable from the immunoprecipitated ^{35}S-labeled p120 protein (Figure 2). This protein was not precipitated by the preimmune IgG. Both ^{32}P- and ^{35}S-labeled precipitates were recognized by the anti-p120 monoclonal antibody. These results show that p120 is a phosphoprotein recognized both by the monoclonal and polyclonal antibodies.

The tryptic peptide map of p120 labeled *in vivo* contained three major phosphorylated peptides (Figure 3A). The cDNA-derived amino acid sequence of p120 (6) showed clusters of acidic residues C-terminal to the serine or threonine which may be phosphorylated by casein kinase II (18, 19). One cluster is located between Ser-181 and Glu-191 (SEEETEDEEEE). This region is at the C-terminal of the epitope recognized by the anti-p120 monoclonal antibody (residues 173-180). A DNA fragment coding for the epitope and this acidic region was inserted into the maltose binding protein gene. The expressed fusion protein

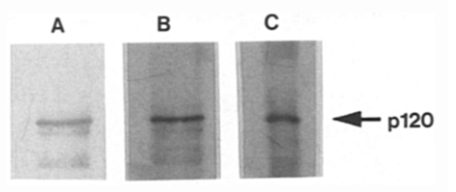


Figure 2. **Analysis of immunoprecipitates from ^{32}P- or ^{35}S-labeled HeLa nuclear extracts.** HeLa cells were labeled with [^{32}P]-orthophosphate or [^{35}S]-methionine and nuclear extracts were subjected to immunoprecipitation using anti-p120 polyclonal antibodies as described in Materials and Methods. Western blot analysis **(A)** of the immunoprecipitates was done using anti-p120 monoclonal antibody. SDS-polyacrylamide gel of ^{32}P-labeled **(B)** and ^{35}S-labeled **(C)** immunoprecipitates showed an antigen with an apparent molecular weight of 120,000 (p120).

(MBP120.21 in Figure 4) had the activity of the maltose binding protein (MBP) for binding to cross-linked amylose. The purified MBP120.21, but not MBP, was phosphorylated *in vitro* by casein kinase II (lanes 1 and 3 in Figure 4C). The tryptic digest of *in vitro* phosphorylated MBP120.21 contained one labeled phosphopeptide (Figure 3B) which comigrated with one of the peptides from the *in vivo* phosphorylated p120 tryptic digest (spot 2 in Figure 3C). This indicates the possibility that HeLa p120 is phosphorylated by casein kinase II in at least one site.

Either Ser-181 or Thr-185 may be phosphorylated by casein kinase II. The phosphoamino acid analysis of the *in vitro* phosphorylated peptide showed that threonine was phosphorylated 4-fold more than the serine residue (Figure 5A). Analysis of the *in vivo* phosphorylated p120 showed it contained phosphoserine, phosphothreonine and phosphotyrosine (Figure 5B).

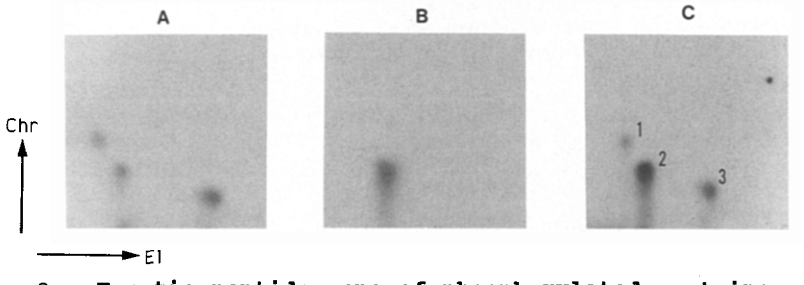
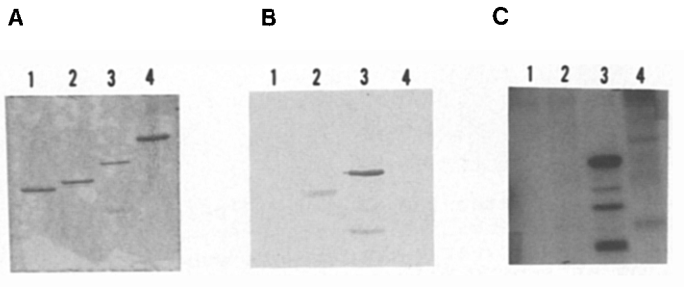
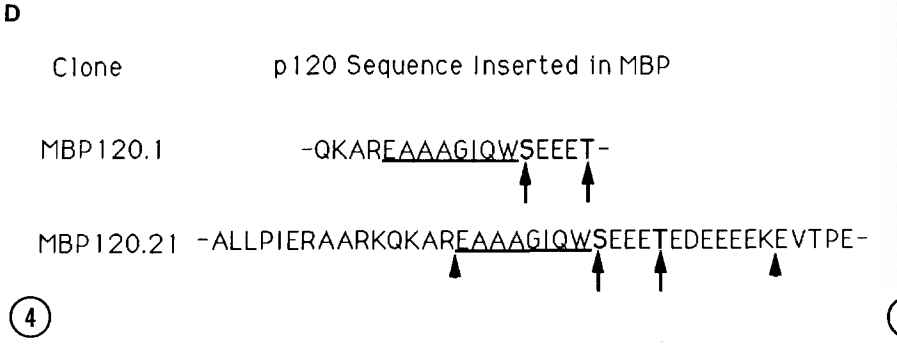


Figure 3. **Tryptic peptide maps of phosphorylated proteins. (A)** *In vivo* phosphorylated p120 was immunoprecipitated from HeLa nuclear extract and treated with trypsin as described in Materials and Methods. Tryptic digests were analyzed by electrophoresis in the horizontal dimension (E1) and by ascending chromatography (Chr). **(B)** Maltose binding protein containing p120 sequence (MBP120.21 in Figure 4) was phosphorylated *in vitro* using casein kinase II, digested with tyrpsin and analyzed as in (A). **(C)** Mixture of the tryptic digests of *in vivo* (A) and *in vitro* (B) phosphorylated proteins.





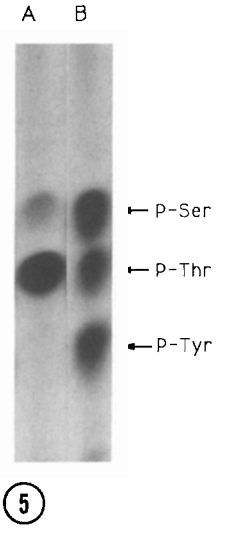


Figure 4. **Phosphorylation of maltose binding protein containing p120 sequences.** The proteins were used as substrates for in vitro phosphorylation by casein kinase II and analyzed by coomassie staining of SDS-polyacrylamide gel **(A)**, Western blot using anti-p120 monoclonal antibody **(B)**, and autoradiography **(C)**. Lanes: 1, maltose binding protein; 2, MBP120.1; 3, MBP120.21; 4, bovine serum albumin. **(D)** Sequence of p120 fragments inserted into a "permissive" site of the maltose binding protein as described in Materials and Methods. The epitope region recognized by the anti-p120 monoclonal antibody is underlined. Arrows show the residues that may be phosphorylated by casein kinase II. Arrow heads show the tryptic digestion sites.

Figure 5. **Phosphoamino acid analysis.** Tryptic digests of in vitro phosphorylated MBP120.21 **(A)** or in vivo phosphorylated HeLa p120 **(B)** were hydrolyzed in 6 N HCl at 110°C for 2 hr under N_2 gas. The hydrolysate was mixed with phosphoamino acid standards and fractionated on cellulose sheets by high voltage eletrophoresis. Amino acids were stained with ninhydrin, and the ^{32}P radioactivity was detected by autoradiography.

DISCUSSION

The full-length p120 cDNA constructed in this study permits rapid studies on the structural characterization and functional identification of this nucleolar antigen which is functionally important in the G1 phase of cancer and proliferating cells (17). Using the bacterially expressed p120, polyclonal antibodies were generated which facilitated further characterization of p120. The present study demonstrates that this proliferation-associated antigen is a phosphoprotein.

To identify the possible site(s) of phosphorylation in p120, casein kinase II was used to phosphorylate the p120 peptide in vitro. Region 181-191 which is C-terminal to the epitope region recognized by the anti-p120 monoclonal antibody was examined to determine if it was phosphorylated and if this modification would affect the binding of the monoclonal antibody to the adjacent epitope. This peptide was expressed by genetic insertion into a "permissive" site of the maltose binding protein (MBP). The hybrid protein retains the properties of the wild type MBP so that purification was possible in one step.

The hybrid protein was phosphorylated with casein kinase II and digestion with trypsin yielded a p120 phosphopeptide (Figure 3B). This phosphopeptide comigrated with one of the three phosphopeptides in the tryptic peptide map of in vivo phosphorylated p120 which indicates the possiblity that HeLa cell p120 is in vivo phosphorylated at Thr-185 and/or Ser-181 by casein kinase II. The phosphorylation on Ser-181 and/or Thr-185 did not affect the binding of the anti-p120 monoclonal antibody (data not shown).

Casein kinase II has been found in high concentration in the nucleolus of mouse tumor cells and it phosphorylates nucleolar proteins like B23, C23 and pp135 (20). Addition of a negatively charged phosphate group in a negatively charged acidic domain may intensify the binding of these proteins to basic proteins.

Figure 5B shows that the in vivo phosphorylated HeLa p120 also contained phosphotyrosine in addition to phosphoserine and phosphothreonine. This implies that not only casein kinase II is involved in the in vivo phosphorylation of p120 but also a tyrosine kinase must phosphorylate p120 in vivo.

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