Identification of an Epitope Region of the Human Proliferation-associated Nucleolar Antigen P120

Benigno C. Valdez, Rose K. Busch, Robert G. Larson, and Harris Busch
Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030

ABSTRACT

An epitope region, located at amino acid residues 173-180 (EAAA-GIQW), of a human cell proliferation-associated nucleolar antigen, P120, has been defined by mutational analysis and competition assays. A synthetic peptide corresponding to this epitope region completely blocks the binding of the anti-P120 antibody to Escherichia coli-expressed P120 and the HeLa nucleolar P120 protein. Adjacent peptides lack inhibitory effects. The antigenic site includes a hydrophilic residue and a hydrophobic stretch. The glutamyl and tryptophanyl residues in this region make major contributions to the binding of P120 to its antibody, since peptides lacking either the glutamyl or the tryptophanyl residue do not block the antibody binding to the P120 antigen. This study provides a basis for drug design for specific binding to the epitope region of the P120 protein.

INTRODUCTION

One important difference between cancer and normal cells is hyperactivity and pleomorphism of the nucleoli (1). Biochemical and immunological analyses have shown elevated levels of some proteins in cancer cell nucleoli, as compared to normal resting cell nucleoli (2-4). The development of polyclonal and monoclonal antibodies against these proteins has led to characterization of several tumor-associated nucleolar antigens. Some of these antigens are involved in cell proliferation (4-8).

A cell proliferation-associated nucleolar antigen, identified in our laboratory with the aid of a monoclonal antibody, has an apparent molecular weight of 120,000 (designated as P120). This antigen was unique in that it was detected in a wide range of malignant tumors but not in most normal (resting) cells. P120 protein was not detected in human colon; other nucleolar antigens including P145 were found in normal colon (7). Moreover, unpublished findings done jointly with the Michigan Cancer Institute indicated that this antigen had prognostic value in breast cancer.5 Quantitative slot blot studies showed there was 60 times more P120 mRNA in human tumor cells than in human term placenta (9).

The P120 protein appears in the nucleolus early in the G1 phase of the cell cycle (7) and has been shown by immunoelectron microscopy to be associated with a network of beaded microfibrillar structures in the nucleolus (10). This observation has led Ochs et al. (10) to suggest that P120 is a binding protein that attaches rDNA and/or preribosomes to the nucleolar matrix, similar to the association of some nuclear oncogene products with the nuclear matrix. The nucleotide and cDNA-derived amino acid sequences of P120 have been determined (11). This protein contains four major domains: a basic domain and an adjacent acidic domain in the amino-terminal portion, a hydrophobic methionine-rich domain in the center of the molecule, and a cysteine-rich domain in the carboxy-terminal portion. The cDNA clone has been shown to code for the HeLa P120 protein by similarities in immunoreactivity, migration on SDS-polyacrylamide gel electrophoresis, and partial digestion peptide maps of the Escherichia coli-expressed P120 and the HeLa cell nucleolar P120 protein (11). Recently, the gene for P120 has been sequenced.6

The proliferation-associated antigens may be important targets for the development of anticancer drugs. Freeman and Bondada (12) showed that scrape-loaded anti-P120 monoclonal antibody inhibited HeLa cell proliferation and DNA and RNA syntheses in a dose-dependent manner. Other monoclonal antibodies to nucleolar protein B23 did not inhibit cell proliferation. These results suggest that an optimal site for inhibition of the functions of the P120 protein is the epitope region recognized by the monoclonal antibody. Furthermore, binding of a monoclonal antibody to activated c-H-ras proteins was shown to inhibit their ability to induce transformation (13, 14). The present study reports the identification of the sequence of an octapeptide epitope of the protein P120.

MATERIALS AND METHODS

Cloning and Expression of Epitope Region. A 700-base pair EcoRI fragment from a fetal liver P120 cDNA (11) coding for the epitope-containing region was cloned at the EcoRI site of pET5A vector (15) (Fig. 1). The SalI-SspI fragment of pET5A was subcloned at the SalI-Smal sites of pTZ19R (United States Biochemical Corporation, Cleveland, OH) for convenience in making deletions. The resulting clone, pTZ5A1, was used to transfect E. coli strain JM105. To express the protein, JM105 harboring pTZ5A1 was grown in Lennox L broth containing 50 μg/ml ampicillin at 37°C and was made to 1 mivI IPTG when the culture reached an A600 of 0.5-0.7. The incubation was continued and the cells were harvested 4 h after induction.

Construction of Deletion Mutants and Peptide Synthesis. Deletion mutants were prepared either by treatment with Exonuclease III, using an Erase-A-Base kit (Promega, Madison, WI), or by the polymerase chain reaction system, using a GeneAmp kit (Perkin-Elmer Cetus, Norwalk, CT). pTZ5A1 was digested with Asp718 and SsrI. 3'-Deletion of the insert was done using an Erase-A-Base kit, following the procedure recommended by the supplier.

The polymerase chain reaction was used to construct deletion mutants as illustrated below:

\[ \text{5'} \] Pr2 \hspace{1cm} Xho1 \hspace{1cm} A \hspace{1cm} B \hspace{1cm} SsrI \hspace{1cm} 3' \]

pTZ5A1

Two synthetic oligonucleotide primers (Pr1 and Pr2) were used to amplify certain regions of the gene with the AB fragment deleted, according to the procedure provided with the GeneAmp kit. The amplified region was cut with XhoI and SsrI and subcloned at the XhoI-SsrI sites of pTZ5A1. This replaced the longer XhoI-SsrI insert in

5' Pr1 \hspace{1cm} Xho1 \hspace{1cm} A \hspace{1cm} B \hspace{1cm} SsrI \hspace{1cm} 3' pTZ5A1

Received 10/27/89; revised 1/8/90.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This investigation was supported by the Cancer Research Center Grant CA-10893, awarded by the National Cancer Institute, Department of Health and Human Services, USPHS; The DeBakey Medical Foundation; The Davidson Fund; The Pauline Sterne Wolff Memorial Foundation; H. Leland Kaplan Cancer Research Endowment; Linda and Ronny Finger Cancer Research Endowment Fund; and The William S. Parish Fund.

2 To whom requests for reprints should be addressed.

3 J. Freeman, R. K. Busch, H. Ownby, and H. Busch, unpublished observations.

4 The abbreviations used are: cDNA, complementary DNA; ELISA, enzyme-linked immunosorbent assay; IPTG, isopropyl-β-D-thiogalactoside; SDS, sodium dodecyl sulfate.


2704
Sail EcoRI construction and expression of human proliferating cell nucleolar antigen pi 20

gel containing 0.1 % SDS, as described above. The gel was transferred in Escherichia coli, submitted for publication.

blocked with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% of these extracts was performed on a preparative 8% polyacrylamide nucleolar extract were used in the competition assays. Electrophoresis

gels with 0.1 % SDS, and electrophoresis was carried out as described previously (18). The gels were stained with Coomassie brilliant blue

described previously (7). Extracts were analyzed on 12% polyacrylamide

Immunology, Baylor College of Medicine, Houston, TX), using a 430A peptide synthesizer (Applied Biosystems, Foster City, CA). Polyacrylamide Gel Electrophoresis and Immunoblotting. E. coli cells

Competition Assay by Western Blotting. An E. coli extract containing P120 protein and HeLa nucleolar extract was done on a preparative SDS-polyacrylamide gel (6 or 8%) and a region corresponding to a molecular weight of 120,000 was excised. Protein P120 was electroeluted (21) from these gel slices. The eluted protein in nanogram quantities was bound to the IMMULON plates (Dynatech Laboratories, Inc., Alexandria, VA) overnight at 4°C. The unbound sites were blocked with 10 mM Tris-HCl, pH 7.5, 0.9% NaCl containing 3% bovine serum albumin and 10% chicken serum. The mouse monoclonal anti-P120 antibody was preincubated with the synthetic peptides for 2 h at room temperature. These samples were then transferred to the IMMULON plate which contained the bound P120. The antibody (plus peptides) was allowed to react with the bound P120 protein for 2 h. The second antibody was a peroxidase-labeled goat anti-mouse IgG. Color development occurred upon the addition of 2,2'-azino-di-3-ethyl-benzthiozolinesulfonic acid, diammonium salt (Boehringer-Mannheim, West Germany). The absorbance was determined at 405 nm on a Microplate Reader (Dynatech) at the end of 15 and 30 min.

RESULTS

Expression of the Epitope-containing Region. Cloning of the 5' half of the P120 cDNA at the EcoRI site of pET5A expression vector (15) placed the cloned cDNA fragment under the control of a T7 promoter (Fig. 1). Transformation of an E. coli strain, BL21(DE3)pLysE, containing an IPTG-inducible T7 RNA polymerase resulted in expression of a polypeptide immunoreactive with the anti-P120 antibody. The Sspl-Sall fragment was subcloned at the SmaI-Sall sites in pTZ19R for production of 3'-deletion mutants. This procedure, which utilized the pET5A and pTZ19R vectors, placed the cloned cDNA under the control of an IPTG-inducible lac promoter. It also used an efficient translation signal (Shine-Dalgarno sequence) from the pET5A vector (Fig. 1) and allowed expression of a protein that reacted with anti-P120 antibody.

Identification of the Epitope Region by Immunoreactivity of Deletion Mutants. Fig. 2 shows the constructed deletion mu-

More deletion mutants were constructed by a similar procedure, using other Pr1 primers closer to the 5' region of the gene. The deletion mutants were sequenced by the dideoxy chain termination method (16), using a Sequenase kit (United States Biochemical Corporation).

Peptides were synthesized by Dr. Richard Cook (Department of Immunology, Baylor College of Medicine, Houston, TX), using a 430A peptide synthesizer (Applied Biosystems, Foster City, CA).

Polyacrylamide Gel Electrophoresis and Immunoblotting. E. coli cells harboring pTZ5A1 were pelleted and lysed by boiling in Laemmli buffer (17). HeLa nucleoli were isolated according to the procedure described previously (7). Extracts were analyzed on 12% polyacrylamide gels with 0.1% SDS, and electrophoresis was carried out as described previously (18). The gels were stained with Coomassie brilliant blue R250 or were transferred to nitrocellulose (19). Immunoreactive bands were visualized using an immunoscreening system (Promega, Madison, WI).

Competition Assay by Western Blotting. An E. coli extract containing P120 protein expressed from a full length P120 cDNA* and a HeLa nucleolar extract were used in the competition assays. Electrophoresis of these extracts was performed on a preparative 8% polyacrylamide gel containing 0.1% SDS, as described above. The gel was transferred on a nitrocellulose filter. Unbound sites on the nitrocellulose filter were blocked with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 3% bovine serum albumin and 10% chicken serum. Monoclonal antibody raised against HeLa P120 (7) was preincubated with the synthetic peptides (final concentration of 1 mM) (Table 1) at room temperature for 2 h and then was allowed to bind to the P120 protein bound on the nitrocellulose filter for 1 h, using a Mini-blotter II (Immunetics, Cambridge, MA). The filter was washed 3 times with TBST (5–10 min each wash), followed by incubation with goat anti-mouse IgG-alkaline phosphatase conjugate, and the immunoreactive bands were visualized using nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt (Promega, Madison, WI).

Competition ELISA Assay. The ELISA procedure was carried out as described previously (20). Electrophoresis of E. coli extract containing expressed P120 protein and HeLa nucleolar extract was done on a preparative SDS-polyacrylamide gel (6 or 8%) and a region corresponding to a molecular weight of 120,000 was excised. Protein P120 was electroeluted (21) from these gel slices. The eluted protein in nanogram quantities was bound to the IMMULON plates (Dynatech Laboratories, Inc., Alexandria, VA) overnight at 4°C. The unbound sites were blocked with 10 mM Tris-HCl, pH 7.5, 0.9% NaCl containing 3% bovine serum albumin and 10% chicken serum. The mouse monoclonal anti-P120 antibody was preincubated with the synthetic peptides for 2 h at room temperature. These samples were then transferred to the IMMULON plate which contained the bound P120. The antibody (plus peptides) was allowed to react with the bound P120 protein for 2 h. The second antibody was a peroxidase-labeled goat anti-mouse IgG. Color development occurred upon the addition of 2,2'-azino-di-3-ethyl-benzthiozolinesulfonic acid, diammonium salt (Boehringer-Mannheim, West Germany). The absorbance was determined at 405 nm on a Microplate Reader (Dynatech) at the end of 15 and 30 min.

Expression of the Epitope-containing Region. Cloning of the 5' half of the P120 cDNA at the EcoRI site of pET5A expression vector (15) placed the cloned cDNA fragment under the control of a T7 promoter (Fig. 1). Transformation of an E. coli strain, BL21(DE3)pLysE, containing an IPTG-inducible T7 RNA polymerase resulted in expression of a polypeptide immunoreactive with the anti-P120 antibody. The Sspl-Sall fragment was subcloned at the SmaI-Sall sites in pTZ19R for production of 3'-deletion mutants. This procedure, which utilized the pET5A and pTZ19R vectors, placed the cloned cDNA under the control of an IPTG-inducible lac promoter. It also used an efficient translation signal (Shine-Dalgarno sequence) from the pET5A vector (Fig. 1) and allowed expression of a protein that reacted with anti-P120 antibody.

Identification of the Epitope Region by Immunoreactivity of Deletion Mutants. Fig. 2 shows the constructed deletion mu-

Immunoactivity

<table>
<thead>
<tr>
<th>Deletion Mutant</th>
<th>Immunoactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTZ5A1</td>
<td>+</td>
</tr>
<tr>
<td>DM253</td>
<td>+</td>
</tr>
<tr>
<td>DM206</td>
<td>+</td>
</tr>
<tr>
<td>DM180</td>
<td>+</td>
</tr>
<tr>
<td>DM171</td>
<td>+</td>
</tr>
<tr>
<td>DM174/186</td>
<td>+</td>
</tr>
<tr>
<td>DM174/180</td>
<td>+</td>
</tr>
</tbody>
</table>

tants and their immunoreactivity with the anti-P120 antibody. Deletion from the 3’ end of the cDNA insert up to nucleotide 541 (or amino acid 181) produced mutants that were immunoreactive with anti-P120 antibody. Further 3’ deletion to nucleotide 514 (or amino acid 172) did not give an immunoreactive mutant. Deletion of amino acid residues 175–180 produced a polypeptide which did not react with the anti-P120 antibody. Based on these constructed mutants, amino acid residues 175–180 (AAAGIQW) are an important part of the epitope region.

**Competition Assay by Western Blotting.** The sequences of the peptides used in the competition assay and their effect on binding of anti-P120 antibody to P120 protein are shown in Table 1 and Fig. 3. Among these peptides, only the epitope peptide (EAAAGIQW) inhibited the binding of the antibody to E. coli-expressed P120 and the HeLa nucleolar P120 protein (Fig. 3). Thus, this peptide probably represents the sequence of the epitope region.

To determine if the tryptophanyl residue was a critical part of the epitope region, a peptide similar to the epitope peptide without tryptophan (peptide A1 in Table 1) was used in the competition assay. No inhibition of antibody binding to P120 was detected using 1 mM concentrations of this new peptide. Similarly, a very weak inhibition was observed when glutamate was removed (peptide A2) from the epitope peptide (Fig. 3). These results show that the epitope region includes amino acid residues 173–180.

**Competition ELISA Assay.** Fig. 4 illustrates the effect of preincubation of various peptides with the P120 monoclonal antibody in an ELISA assay. The epitope peptide inhibits the binding of the antibody to the antigen with an IC$_{50}$ of approximately 5 nM. The binding of the E. coli-expressed P120 protein to the P120 antibody dropped from 100% to 4% with 32 nM epitope peptide (Fig. 4). The other peptides had little or no effect. Similar results were obtained when the electroeluted HeLa P120 was bound to the ELISA plate (data not shown); these results agree well with the deletion and Western blot competition experiments.

**DISCUSSION**

Based on deletion mutations, an important part of the epitope region of P120 protein that binds to the monoclonal anti-P120 antibody (7) included amino acid residues 175–180 (AAAGIQW). Competition assays confirmed this result. Since the peptide corresponding to amino acid residues 175–180 had a very low solubility in water, a synthetic peptide corresponding to amino acid residues 173–180 (EAAAGIQW) was used in the competition assays. Western blot and ELISA procedures (Figs. 3 and 4) showed that preincubation of the anti-P120 antibody with the epitope peptide (Table 1) blocked the binding of the antibody to E. coli-expressed P120 and the HeLa nucleolar P120 protein, but adjacent peptides (triple E and signal peptides) lacked inhibitory effects. The inhibition by the epitope peptide was specific for the P120 protein, since preincubation of the anti-B23 antibody with the epitope peptide did not block binding to B23 protein (21, 22) (data not shown). Deletion of either the tryptophanyl or the glutamyl residue in the epitope peptide prevented inhibition of the binding of the P120 protein to its antibody. These results show that the epitope region is located at amino acid residues 173–180, two amino acid residues longer than the region identified by mutations. The competition assays provided similar results for both E. coli-expressed P120 protein and the HeLa nucleolar P120 protein, which supports the recent findings that the cDNA clone we isolated codes for the HeLa P120 protein (11).

The identified epitope is limited to a small region of P120. This immunogenic site is a “continuous antigenic site” which occupies the surface region of the antigen and constitutes a continuous part of the polypeptide chain (23). The site is small.
EPITOPE REGION OF A PROLIFERATION-ASSOCIATED ANTIGEN

(eight amino acid residues) but smaller size antigenic sites (six or seven amino acid residues) have been identified in sperm whale myoglobin (24).

Examination of the structure of the epitope (EAAAGIQW) shows a hydrophobic stretch (AAAGIQW) with a hydrophilic residue on one side. It is likely that glutamate and tryptophan in the identified epitope region bind to the antibody and the other residues contribute to a lesser extent to the binding. The inability of tetraalanine peptide to inhibit the immunogenic activity of P120 supports the hypothesis that the three alanine residues are not major contributors to the P120-antibody interaction.

The epitope region is located between two acidic clusters (amino acids 151–157 and 182–191). These acidic sequences are probably part of a nucleolar recognition signal, since they are also present (60–85% homology) in nucleolar phosphoproteins B23 (25) and C23 (26). The importance of the epitope region for the function of P120 is shown by the inhibition of cell proliferation and DNA/RNA synthesis when anti-P120 antibody is microinjected into HeLa cells (12). This result provides evidence that binding of a drug to the epitope region may have antiproliferative effects in cancer.

We are presently performing site-directed mutagenesis to identify the amino acid residues directly involved in the binding of P120 to its antibody. The results from this work and a possible three-dimensional image will provide a better picture of the epitope structure.

REFERENCES


Identification of an Epitope Region of the Human Proliferation-associated Nucleolar Antigen P120


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/50/9/2704

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.