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Product of the Oncogene-Activating Gene *Tpr* Is a Phosphorylated Protein of the Nuclear Pore Complex

Peter L. Bangs, Cynthia A. Sparks, Paul R. Odgren, and Edward G. Fey

Department of Cell Biology, University of Massachusetts Medical Center, Worcester, Massachusetts 01655

Abstract We have identified a component of the human nuclear pore complex and have shown that it is the product of a gene involved in oncogenic activation. A monoclonal antibody raised against purified nuclear matrix proteins recognizes a single protein with an electrophoretic mobility of approximately 300 kDa and stains the nuclear envelope in a punctate pattern typical of nuclear pores. The antibody was used to screen λgt11 human cDNA libraries, and the resulting clones were sequenced and compared to sequences in the Genbank database. An exact match was found with the human *tpr* (for translocated promoter region) gene, a gene shown previously to be involved in the oncogenic activation of several protein kinases. Double-label immunofluorescent microscopy with the anti-Tpr antibody and an antibody to the previously characterized nuclear pore complex protein nup153 confirms that Tpr is localized to the nuclear pore complex. Tpr is located on the cytoplasmic face of the nucleus, as demonstrated by immunofluorescent staining of cells permeabilized with digitonin. Tpr is a 2,349-amino acid protein with extensive coiled-coil domains and an acidic globular C-terminus. The protein contains 10 leucine zipper motifs and numerous sites for phosphorylation by a variety of protein kinases. Immunoprecipitation of Tpr from ^{32}P-orthophosphate-labeled cells shows that it is a phosphoprotein. Potential functions for Tpr and possible mechanisms for the transforming activity of Tpr fusion proteins are discussed.

Key words: nuclear pore structure, digitonin permeabilization, immunofluorescence, coiled-coil proteins, *Tpr*

The nuclear pore complex (NPC) is a large organelle that joins the inner and outer membranes of the nuclear envelope and allows the passive diffusion of ions and small molecules while regulating the nucleocytoplasmic transport of larger proteins and ribonucleoprotein particles. A supramolecular assembly of approximately 125 MDa, the NPC is roughly 130 nm in diameter and 70 nm thick [Akey, 1989; Reichelt et al., 1990]. It consists of a central channel complex enveloped by a modular assembly with eightfold rotational symmetry composed of eight multidomain spokes sandwiched by a cytoplasmic and nuclear ring [Akey, 1989; Akey and Radermacher, 1993; Hinshaw et al., 1992; Jarnik and Aebi, 1991; Pante and Aebi, 1993]. Distinct filamentous structures are attached to each of the two rings: a series of eight short filaments decorate the cytoplasmic ring, while the nuclear ring has eight 50- to 100-nm filaments joined distally by a terminal ring to form a "cage" or "basket" structure [Goldberg and Allen, 1992; Jarnik and Aebi, 1991; Pante and Aebi, 1993; Pante et al., 1994]. The proteins that make up the NPC are collectively referred to as nucleoporins (nups).

Of the estimated 100 or more distinct proteins that are believed to comprise the NPC, relatively few have been identified and characterized at the primary structure level [Rout and Wente, 1994]. Among these is a family of 10 or more glycoproteins that have been identified as components of the vertebrate NPC [Davis and Blobel, 1987; Holt et al., 1987; Snow et al., 1987]. These proteins are modified with O-linked N-acetylglucosamine and all have multiple copies of a degenerate peptide motif, FXFG. Several yeast NPC proteins have also been characterized that contain the FXFG repeats [Davis and Fink, 1990; Rout and Wente, 1994] demonstrating the evolutionary conservation of this motif. Another family of yeast nucleoporins has been discovered and characterized that contains multiple copies of a GLFG motif [Fabre et al., 1994; Wente and Blobel, 1994; Wente et al., 1992; Wimmer et al.,

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Address reprint requests to Peter L. Bangs, Department of Cell Biology, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01655.

1992]. The recent characterization of a mammalian nucleoporin with GLFG repeats [Radu et al., 1995] provides further evidence of the structural conservation of nuclear pore proteins.

Several nucleoporins contain conserved motifs that suggest possible functions for these proteins. Nup153 contains four zinc finger motifs and has been shown to bind DNA in a zinc-dependent fashion in vitro [Pante et al., 1994; Sukegawa and Blobel, 1993]. This finding is consistent with the proposal that nucleoporins may play a role in the three-dimensional organization of chromatin as well as function in nucleocytoplasmic transport [Blobel, 1985]. Nup107 has a leucine zipper in its C-terminus, suggesting dimerization and the formation of either homo- or heteromers as part of its function [Radu et al., 1994]. The conserved GLFG repeats found in several yeast and one mammalian nucleoporin have been implicated in the "docking" of substrates prior to transport through the nuclear pore [Radu et al., 1995].

A number of nucleoporins have been unequivocally localized to distinct regions within the NPC. Among these are nup153, which has been firmly established as a component of the intranuclear filaments attached to the nuclear periphery of the NPC [Cordes et al., 1993; Pante and Aebi, 1993; Pante et al., 1994; Sukegawa and Blobel, 1993], and nup180, which has been localized to the cytoplasmic ring of the NPC and could also be a part of the cytoplasmic filaments [Wilken et al., 1993]. Another nucleoporin, nup214, has also been recently localized to the cytoplasmic face of the NPC [Kraemer et al., 1994; Pante et al., 1994].

The specific functions of individual nucleoporins are unknown, but much work has been done to elucidate their role in nucleocytoplasmic transport. Antibodies to several nucleoporins have been shown to inhibit nuclear transport, although it is unclear whether the observed inhibition is due to the specific hindrance of the transport function of these proteins or the result of nonspecific steric affects [Dabauvalle et al., 1990; Featherstone et al., 1988]. Antibodies to at least one NPC protein, the recently identified nup180, do not interfere with nuclear transport [Wilken et al., 1993].

Nucleoporins have also been shown to be involved in the process of oncogenic activation. Nup214 was reported to be a homologue of the human CAN protein [Kraemer et al., 1994], a putative oncogene product involved with myeloid leukemogenesis [von Lindern et al., 1992a]. Two distinct chromosomal aberrations have been identified that result in chimeric proteins composed of the C-terminal portion of nup214 fused to the N-termini of the DEK and SET proteins [von Lindern et al., 1992a,b]. It has been suggested that nup214 is the oncogene product, rather than the DEK and SET proteins [von Lindern et al., 1992b], because nup214 is the common element in both fusions. The function of nup214 is unknown; one suggested possibility is that it serves as a docking site for receptor-mediated import of substrates through the NPC [Kraemer et al., 1994; Pante et al., 1994]. A disruption in the control of substrate binding brought about by the oncogenic fusion proteins could lead to an imbalance in nucleocytoplasmic traffic control and conceivably yield a proliferative advantage to the affected cell [Kraemer et al., 1994].

Here we report the identification and molecular cloning of another nucleoporin involved in oncogenic transformation. Sequence analysis identifies this protein as the product of the human oncogene-activating gene *tpr* (translocated promoter region) [Byrd et al., 1994; Mitchell and Cooper, 1992] which was originally shown to activate the *met* oncogene through a chromosomal translocation that results in the 5′ portion of *tpr* becoming fused to the 3′ region of *met* [Park et al., 1986]. A recent report has shown that the product of the rat *tpr* gene is a nucleoporin that localizes to the cytoplasmic face of the NPC [Byrd et al., 1994]. Using somewhat different methodologies, we have characterized the human Tpr and confirmed its identity as a protein of the NPC. We show that this protein is a nucleoporin by virtue of its co-localization with a previously characterized protein of the NPC and that it is localized to the cytoplasmic face of the nuclear envelope. The cDNA sequence reported for *tpr* predicts a polypeptide with an extended coiled-coil domain, a globular highly acidic C-terminus, several leucine zipper motifs, and numerous phosphorylation sites for a variety of kinases. We show here that the protein encoded by human *tpr* is a phosphoprotein with an electrophoretic mobility of approximately 300 kDa.

MATERIALS AND METHODS

Monoclonal Antibodies

Monoclonal antibodies (mAbs) were prepared by Matritech (Cambridge, MA). Nuclear matrix

proteins were isolated as described [Fey and Penman, 1988] from the Caski human cervical tumor cell line. Briefly, cells were extracted with nondenaturing detergents, digested with both RNase A and DNase I, and the chromatin removed by ammonium sulfate. The resulting nuclear matrix-intermediate filament scaffolds were dissolved in disassembly buffer containing urea and β-mercaptoethanol. Intermediate filaments were repolymerized by dialysis against a reducing KCl/imidazole buffer and were then removed by ultracentrifugation. The supernatant, greatly enriched in nuclear matrix (NM) proteins, was used for the production of monoclonal antibodies. Balb/c by J mice (Jackson Laboratory, Bar Harbor, ME) were injected intraperitoneally with purified Caski NM protein every 2 weeks, for a total of 16 weeks. Spleen cells were fused with the SP2/O-Ag14 mouse myeloma line, using the method of Koehler and Milstein [Koehler, 1975]. Hybridomas producing antibodies that reacted with nuclear matrix proteins were cloned and grown as ascites. One clone, mAb 203.37, was selected that reacted with a 300-kDa protein on Western blots.

Library Screening

Clones for Tpr were obtained from a λgt11 human breast carcinoma cDNA expression library (Clontech, Palo Alto, CA). Library screening was carried out according to the manufacturer's instructions. Eight independent clones were obtained with mAb 203.37. The cDNA inserts were amplified by polymerase chain reaction (PCR), using primers specific for the λgt11 cloning site and subsequently subcloned into the pCRII vector (Invitrogen, Madison, WI). Further clones were obtained by screening a λ Uni-Zap HeLa cDNA library (Stratagene, La Jolla, CA) with a digoxygenin-labeled probe derived from the previous Tpr cDNA clones. Positive phage clones were converted to pBLUESCRIPT-based plasmids by the plasmid rescue procedure following manufacturer's instructions.

Sequencing

DNA sequences were obtained using the dideoxy method of Sanger et al. [1977]. Sequencing reactions were done with double-stranded templates using the *fMol* DNA Sequencing System (Promega, Madison, WI). Sequencing reactions were primed with the pBLUESCRIPT vector-specific T7 and T3 primers, as well as primers specific to internal sequences in the cDNA clones.

Northern Analysis

Northern blots were performed essentially as described by Maniatis et al. [1982]. Polyadenylated RNA (15 μg) isolated from the ME180 cell line was resolved on a formaldehyde gel. RNA was transferred to nylon membranes and probed with ^{32}P-labeled clone λg203-F. After washing, filters were autoradiographed on Kodak X-ray film. To detect the TPR mRNA, typical exposures were 2–14 days.

Immunofluorescence Microscopy

Cell extraction and fixation. ME-180 human cervical carcinoma cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) on glass coverslips and washed twice with phosphate-buffered saline (PBS). Nonextracted cells were fixed at 4°C for 15 min in PBS containing 3.7% formaldehyde. Cells were washed 3 times in PBS, then permeabilized with PBS containing 0.1% Triton X-100 for 1 min. Some cells were extracted with cytoskeleton buffer (CSK: 300 mM sucrose, 100 mM NaCl, 10 mM Pipes, 3 mM $MgCl_2$, 1 mM EGTA, and 0.5% Triton X-100, pH 6.8) [Fey et al., 1984] for 1 min at 4°C and fixed for 15 min on ice with 3.7% formaldehyde in CSK.

Digitonin permeabilization. Cells were extracted with digitonin according to the method of Adam et al. [1990]. ME180 human cervical carcinoma cells were grown in DMEM supplemented with 10% FBS. Cells grown on glass coverslips were washed twice with PBS and permeabilized in 0.004% digitonin in PBS for 5 min at 4°C. Extracted cells were fixed with 3.7% formaldehyde in PBS for 15 min on ice.

Fluorescent labeling of cells. Following fixation, cells were washed with PBS and with PBS with 0.5% bovine serum albumin (PBSA). Monoclonal antibodies to Tpr (mAb 203.37) and to lamin B (101.B7) were from Matritech, Cambridge, MA. The anti-nup153 antibody mAb 322 was provided by Gunter Blobel (Rockefeller University, New York, NY). Antibodies were used in conjunction with fluorochrome-conjugated goat or donkey anti-IgG secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA). Directly fluoresceinated mAb 203.37 was obtained from Matritech. Antibodies were diluted in PBSA, overlaid with coverslips on sheets of parafilm, and incubated at 37°C. Coverslips were washed three times, 5 min per wash, in PBSA. Secondary antibodies were added and

incubated as above. Following the final antibody incubations, coverslips were washed four times in PBSA containing 0.1% Triton X-100 and one time in PBSA with the DNA counterstain DAPI (14 μM). Finally, the coverslips were washed once with PBSA, twice with PBS, and then mounted with 90% glycerol containing the antifading agent phenylenediamine.

In the case of double antibody labeling with directly fluoresceinated mAb 203.37, cells were stained with either the anti-nup153 antibody mAb 322 or the antilamin B antibody mAb 101.B7, followed by rhodamine-conjugated secondary antibody as described above. The cells were then postfixed with 3.7% formaldehyde in PBS for 15 min at room temperature, washed three times with PBS, and once with PBSA. The cells were then incubated with 1.4 mg/ml mouse IgG in PBSA for 1 h at 37°C, to block remaining mouse IgG-specific binding sites on the rhodamine-conjugated secondary antibodies. Cells were then incubated for 1 h at 37°C with directly fluoresceinated mAb 203.37 in PBSA with 1.4 mg/ml mouse IgG. Finally, the cells were washed, stained with DAPI, and mounted on glass coverslips as described above. As controls for artifactual staining induced by these procedures, we repeated these experiments omitting either the primary antibodies or the rhodamine-conjugated secondary antibodies, or both. In all cases, the staining patterns produced by the directly fluoresceinated mAb 203.37 were not altered. Finally, to verify that the secondary antibody was not "recruiting" mAb 203.37 to its location, we used a mouse monoclonal antibody to a cytoplasmic protein as the primary antibody (instead of anti-nup153 or antilamin b antibodies). We could detect no cytoplasmic staining by the directly fluoresceinated mAb 203.37.

Photomicrography

Cells labeled as above were visualized using a Zeiss Axiofot microscope equipped with epifluorescence filters. Images were recorded with a cooled charge-coupled device (CCD) camera (Photometrics Inc.) with either a ×63 or a ×100 objective and multiple bandpass filter sets. For the serial optical sections shown in Figure 2E, an image restoration (deconvolution) algorithm was used to remove out-of-focus light [Carter et al., 1993]. Images were photographed on Ektachrome film (100 ASA, Kodak, Rochester, NY), with exposure times of 2–60 secs.

Total Protein Extraction

ME-180 human cervical carcinoma cells cultured in DMEM supplemented with 10% FBS were washed in PBS, then solubilized in RIPA buffer (NaCl, 150 mM; NP40, 1%; deoxycholate, 0.5%; sodium dodecyl sulfate [SDS], 0.1%; Tris, pH 8.0, 50 mM) containing proteinase inhibitors (bestatin, 40 μg/ml; pepstatin, 0.7 μg/ml; phenylmethylsulfonyl fluoride, 50 μg/ml; aprotinin, 2 μg/ml; leupeptin, 2 μg/ml; E64, 1 μg/ml: Boehringer Mannheim, Indianapolis, IN) for 10 min at 4°C. Insoluble debris was pelleted at 14,000*g* for 30 min at 4°C. Protein concentrations were determined by the Coomassie Plus microassay (Pierce Chemical, Rockford, IL) standardized with BSA.

Western Blot Analysis

Western blots were performed according to the method of Towbin et al. [1979]. Briefly, polyacrylamide gels were run according to the method of Laemmli [1970]. Proteins were transferred onto PVDF membrane (Millipore, Bedford, MA) using the semidry blotting technique of Kyhse-Anderson [1984]. The membranes were incubated with antibodies, and reactive bands were visualized according to the manufacturer's instructions for the Immun-Lite chemiluminescent system (BioRad, Richmond, CA).

Immunoprecipitation From ^{32}P-Orthophosphate-Labeled Cells

ME180 human cervical carcinoma cells were cultured at 37°C for 12 h in phosphate-free DMEM (Gibco-BRL/Life Technologies, Gaitherburg, MD) supplemented with 10% dialyzed FBS and 2 mCi/ml ^{32}P-orthophosphoric acid (Dupont/NEN, Boston, MA). The cells were rinsed with PBS and solubilized in RIPA with proteinase inhibitors for 10 min on ice. Insoluble debris was pelleted at 14,000*g* for 30 min at 4°C. The supernatant was precleared of nonspecifically binding materials by incubating twice for 1 h each with 100 μl protein G–Sepharose beads (Pharmacia, Uppsala, Sweden) diluted 1:5 in RIPA buffer with proteinase inhibitors. The precleared supernatant was incubated with 5 μl mAb 203.37 ascites for 6 h at 4°C, followed by 200 μl protein G–Sepharose beads overnight. The beads were pelleted and washed 4 times with 0.5 ml RIPA buffer with proteinase inhibitors prior to SDS–PAGE.

RESULTS

In order to identify and characterize components of the nuclear matrix-intermediate filament scaffold [Fey et al., 1984], a series of mice was immunized with nuclear matrix protein fractions purified from the Caski human breast carcinoma cell line. One of the hybridomas obtained from this study, designated mAb 203.37, produced an antibody that detected a single band with an electrophoretic mobility of approximately 300 kDa on Western blots of human cell protein extracts (Fig. 1). The protein recognized by mAb 203.37 was detected in a variety of human cell lines and tissue types but was not detected on immunoblots of proteins from several nonhuman sources including hamster, mouse, and *Xenopus,* suggesting that mAb 203.37 is specific for an epitope that is not well conserved. The protein was resistant to extraction by Triton X-100, remained insoluble following dissolution of the actin cytoskeleton with ammonium sulfate and was not removed by digestion with RNase and DNase (not shown). The monoclonal antibody 203.37 routinely reacted with a single polypeptide on Western blots indicating that this antibody is specific for a single protein component of the human nuclear matrix/intermediate filament fraction.

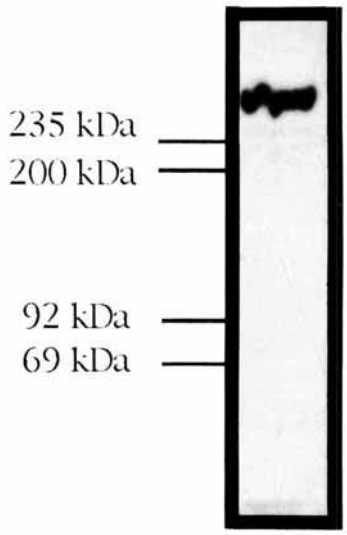

Fig. 1. Immunoblot analyses of the human cervical carcinoma cell line ME180 show that monoclonal antibody 203.37 is specific for a single polypeptide with an approximate electrophoretic mobility of 300 kDa. Cells were lysed with RIPA buffer supplemented with proteinase inhibitors and analyzed by Western blotting using mAb 203.37 and a secondary antibody conjugated to alkaline phosphatase. Proteins were detected by chemiluminescence. Positions of molecular weight markers are shown at left.

Molecular Cloning of Tpr

To better understand the structure of the protein recognized by mAb 203.37, and perhaps deduce its function, molecular characterization of the cDNA encoding this protein was undertaken. The antibody mAb 203.37 was used to screen a λgt11 human breast carcinoma cDNA expression library. Eight independent clones were isolated, and their inserts were sequenced. The inserts ranged in size from 800 to 2,500 base pairs (bp), shared the same 3′ terminus, and differed only in the length of their extensions toward the 5′ end (summarized in Fig. 2A). Since the size of the RNA in Northern blot analyses using the λgt11 clones was approximately 9 kb (Fig. 2B), we screened a λ Uni-Zap HeLa cDNA library to obtain the entire coding sequence. Of the nine clones isolated, five overlapped to form a contiguous sequence extending for more than 7 kb (Fig. 2A).

Nucleotide sequences obtained from the cDNA clones were used to search the EMBL/Genbank data base to find homologous or related sequences. An exact match was found with the oncogene-activating gene *tpr* (Fig. 2C). The cloning and sequencing of the full-length *tpr* cDNA has been reported [Byrd et al., 1994; Mitchell and Cooper, 1992]. The full-length coding sequence for *tpr* consists of a 7,047-bp open reading frame contained within a transcript that spans approximately 9 kb. The product of this cDNA is a 2,349-amino acid polypeptide with a predicted molecular weight of 265 kDa [Byrd et al., 1994]. Given the sequence identity between the cDNA sequence isolated with mAb 203.37 and *tpr,* the matching sizes of the mRNA encoding Tpr and the protein recognized by mAb 203.37, and the similarity between the observed electrophoretic mobility of this protein and that predicted for Tpr, we conclude that the protein recognized by mAb 203.37 is the *tpr* gene product.

Tpr Is a Nuclear Pore Complex Protein

The subcellular location of Tpr was determined by indirect immunofluorescent microscopy. As shown in Figure 3, mAb 203.37 stained the nuclear surface of cells in a punctate pattern that resembles the patterns observed for nuclear pores (e.g., see Sukegawa and Blobel [1993]). The staining pattern did not change with different fixation methods (methanol, acetone, or

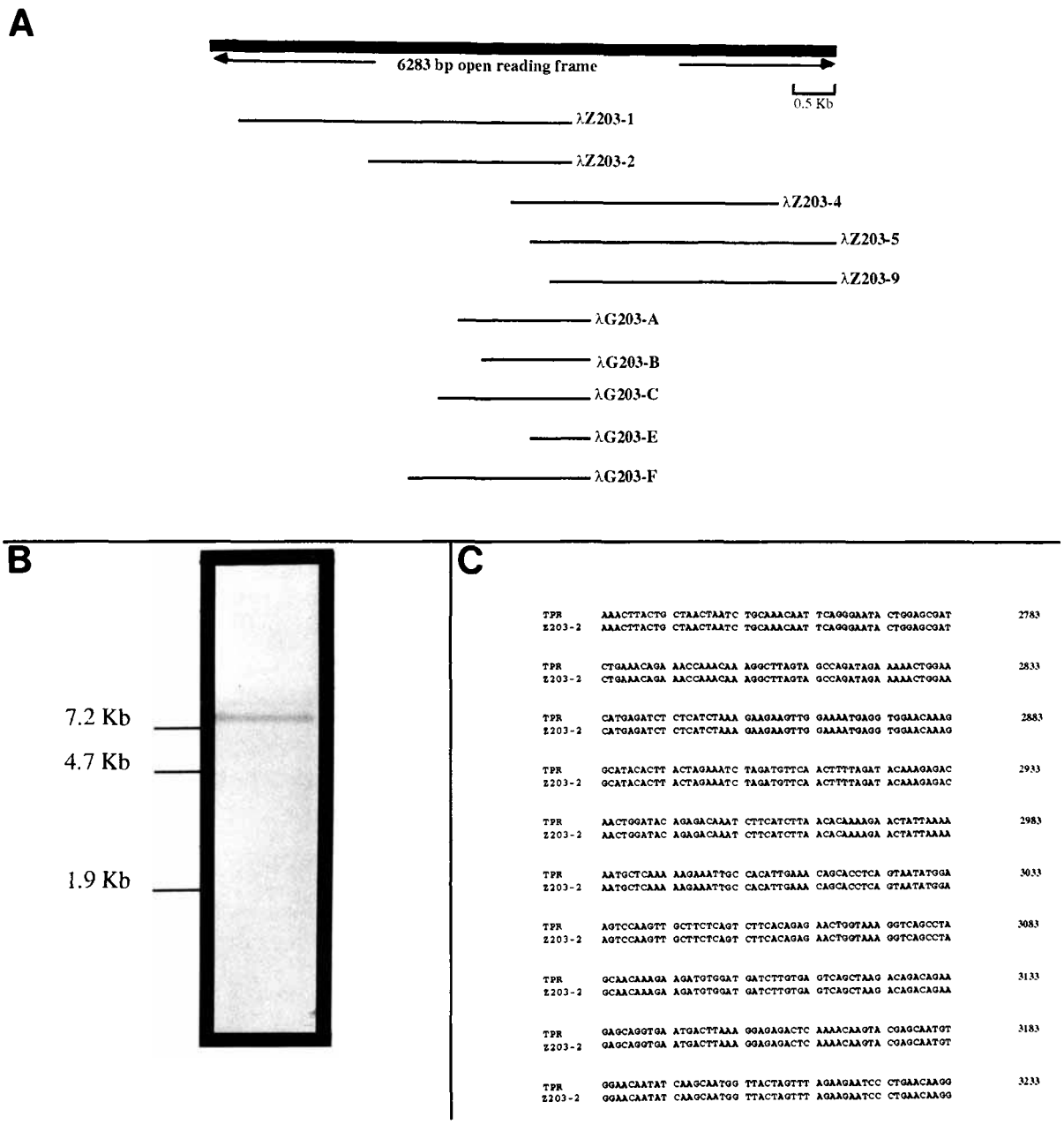

Fig. 2. Monoclonal antibody 203.37 recognizes the product of the human *tpr* gene. The monoclonal antibody 203.37 was used to screen a λgt11 human breast carcinoma cDNA library. Five of the cDNA clones isolated in this manner are shown in **A**: λG203-A, λg203-B, λg203-C, λg203-E, and λG203-F. The largest of these clones, λg203-F, was used to probe a λ Uni-Zap HeLa cDNA library. Nine clones were isolated in this manner, and five of them are also shown in **A** (λZ203-1, λZ203-2, λZ203-4, λZ203-5, and λZ203-9). The clone λg203-F was used to probe Northern blots of human poly A^+ RNA and detected a major band at approximately 9 kb with a fainter band that is somewhat larger (**B**). Several sections of nucleotide sequence obtained from each of the cDNA clones were compared to the GenBank DNA database. Each of the clones was shown to be components of the full-length cDNA reported for human *tpr*. **C**: Alignment between the *tpr* cDNA and a 500-bp section from cDNA clone λZ203-2. The homology is exact.

formaldehyde) or when cells were extracted with nonionic detergents prior to fixation, a characteristic common to proteins of the NPC [Forbes, 1992]. To confirm that staining was localized strictly to the surface of the nucleus, we employed digital imaging microscopy to obtain serial optical sections through nuclei stained with mAb 203.37 (Fig. 3E). When the plane of focus is at the bottom or top of the nucleus, the staining pattern is finely punctate on the nuclear sur-

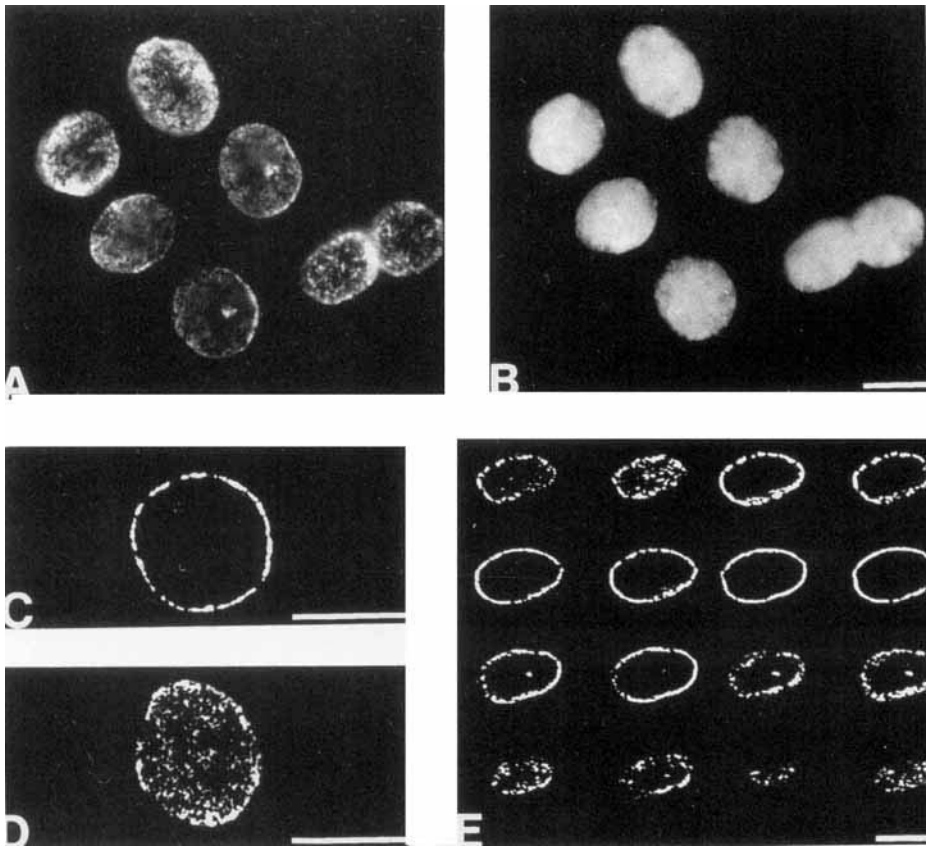

Fig. 3. Indirect immunofluorescent staining of the human cervical carcinoma cell line ME180 demonstrates that mAb 203.37 recognizes a protein localized to the nuclear envelope. Cells grown on glass coverslips were fixed with formaldehyde, permeabilized with 0.1% Triton X-100, and stained with mAb 203.37, followed by fluorescein-conjugated secondary antibody and the DNA-specific stain DAPI. **A:** Field of cells stained in a punctate pattern at the nuclear surface. **B:** DAPI stain of the same field. Slightly higher magnification at different focal planes more clearly demonstrates the punctate localization of mAb 203.37 staining at the nuclear rim **(C)** and at the surface of the nucleus **(D).** Optical sections taken at 0.5-μm intervals through a single nucleus **(E)** show that mAb 203.37 stains predominantly at the nuclear envelope. One or two internal foci are occasionally observed. Scale bars = 10 μm.

face. In the center of the nucleus, the staining pattern is seen along the nuclear "rim" (Fig. 3E, middle panels). These data show that, with the occasional exception of one or two foci located in the nuclear interior (Fig. 3E, panels 9–12), staining is seen exclusively at the nuclear surface.

The punctate staining pattern observed for Tpr suggests that this protein is a component of the NPC. To examine this possibility, we undertook a series of double-label immunofluorescence experiments with antibodies to known proteins of the nuclear lamina and the nuclear pore complex. Initially, cells were stained with antibodies to the nucleoporin nup153 [Sukegawa and Blobel, 1993]. The cells were then incubated with rhodamine-conjugated secondary antibody to visualize nup153 followed by directly fluoresceinated mAb 203.37 to observe Tpr (see Materials and Methods). The staining patterns for the two antigens were largely coincident at the nuclear surface when the nucleus was viewed en face (Fig. 4A,B) or in optical cross sections (Fig. 4C,D) and differed primarily in their staining intensities at different foci. Sometimes the staining intensities in areas of co-localization were in different focal planes suggesting that the antigens were on different sides of the nuclear envelope (see below). The staining pattern of the nuclear lamina protein lamin B did not coincide with Tpr in double-label immunofluorescence experiments (Fig. 4E,F). The lamin B antibody decorated the nuclear surface in a smooth, evenly dispersed fashion, while Tpr staining remained punctate with a nonuniform distribution.

To determine on which side of the nuclear membrane Tpr is located, we used digitonin to selectively permeabilize the plasma membrane while leaving the nuclear membrane intact

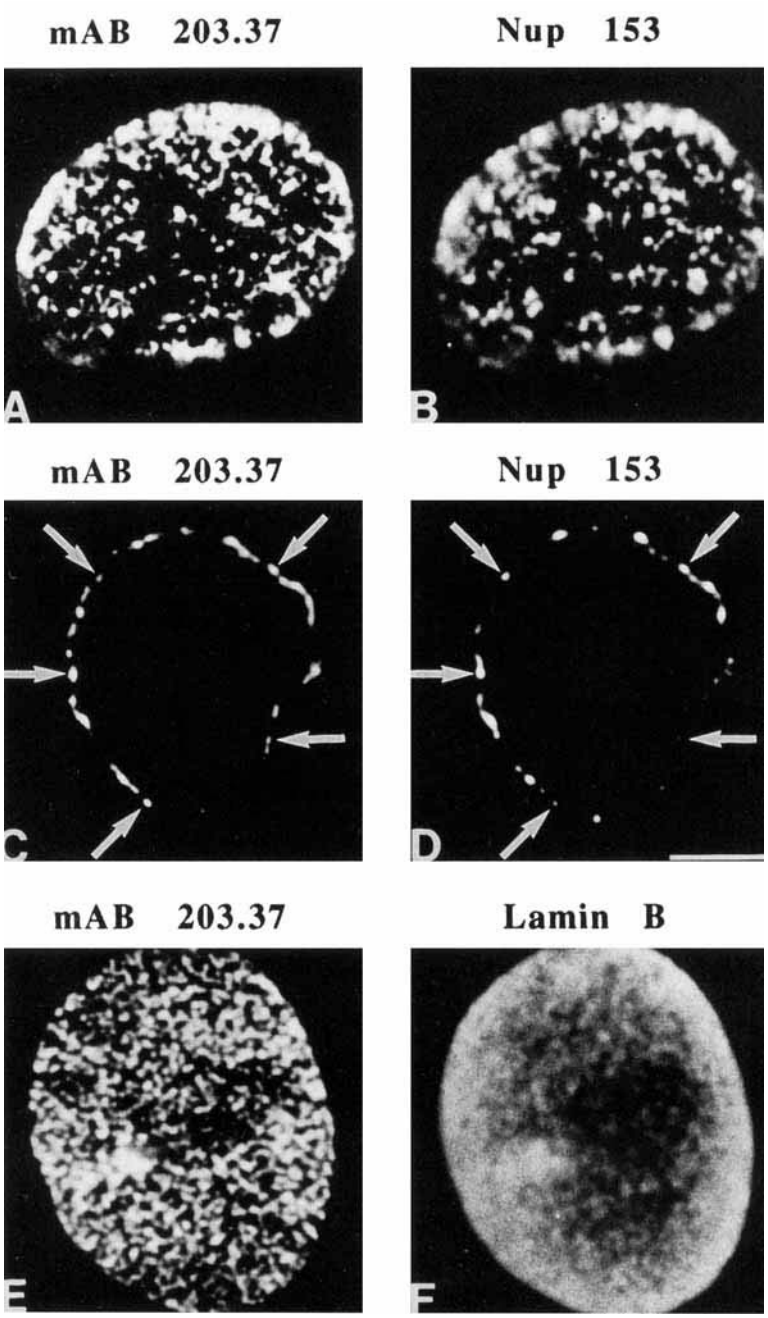

Fig. 4. Tpr co-localizes with the nuclear pore complex protein nup153. Immunofluorescent microscopy of the human carcinoma cell line ME180. After fixation with formaldehyde and permeabilization with Triton X-100, cells were stained with a combination of directly fluoresceinated mAb 203.37 and the anti-nup153 antibody mAb 322 (**A–D**) or with directly fluoresceinated mAb 203.37 and the antilamin B antibody 101.B7 (**E,F**). The antibodies mAb 322 and mAb 101.B7 were detected with rhodamine conjugated secondary antibodies. **A,B:** Surface of a nucleus photographed using filters to show fluorescein staining to detect Tpr (**A**) and rhodamine staining to detect nup153 (**B**). The staining patterns observed for each antibody are largely coincident, differing primarily in the staining intensity at particular foci. **C,D:** Co-localization between Tpr and nup153 is more easily seen, with the rim of a nucleus depicting Tpr (**C**) and nup153 (**D**). *Arrows,* several examples where the antigens are co-localized. **E:** Nucleus stained with mAb 203.37. **F:** Same nucleus stained with mAb 101.B7, demonstrating that the staining patterns for Tpr and lamin B are easily distinguished from one another. Scale bar = 10 μm.

[Adam et al., 1990; Pante et al., 1994]. Under these conditions, the characteristic staining pattern for Tpr was seen (Fig. 5B,C) while no signal was detected with antibodies to either nup153 (Fig. 5D,E) or lamin B (Fig. 5H,I), both of which are located on the nucleoplasmic side of the nuclear membrane. By contrast, when the nuclear membrane was permeabilized with Triton X-100, all three antigens were visualized (Fig. 5A,D,G). These results demonstrate that Tpr is located on the cytoplasmic side of the nuclear membrane.

Tpr Is a Phosphoprotein

Sequence analysis shows that Tpr contains *consensus* sequences for phosphorylation by several protein kinases, including tyrosine kinases, cAMP- and cGMP-dependent protein kinases, protein kinase C (PKC), and casein kinase II [Mitchell and Cooper, 1992]. To determine whether Tpr is a phosphoprotein, we used mAb 203.37 to immunoprecipitate Tpr from cultured human cells labeled with ^{32}P-orthophosphate. A single band of approximately 300 kDa was resolved following SDS–PAGE and autoradiography (Fig. 6). Colorimetric development of the same blot using mAb 203.37 (not shown) confirmed that the labeled band was in fact Tpr and not another phosphoprotein precipitated in association with Tpr.

DISCUSSION

The nucleic acid sequence for the human *tpr* gene was first reported in 1992 [Mitchell and Cooper, 1992]. Based on regions of homology to known nuclear proteins, it was suggested at that time that *tpr* may encode a nuclear protein. In this report, we have confirmed that the product of the *tpr* gene is a component of the nuclear pore complex. Using a monoclonal antibody specific for human Tpr, we have shown that Tpr is a phosphorylated protein with an electrophoretic mobility of approximately 300 kDa, making Tpr the largest nuclear pore complex protein characterized to date.

As reported previously [Mitchell and Cooper, 1992], the N-terminal 80% of Tpr has extensive α-helical regions of heptad repeats, an arrangement that places predominantly hydrophobic residues along one face of the helix. In other proteins, similar motifs promote the formation of a coiled-coil structure between neighboring α-helices, encouraging the establishment of rod-like structures and facilitating protein homo- and/or heteropolymerization stabilized by hydrophobic forces [Cohen and Parry, 1990]. The C-terminal portion of Tpr is extremely acidic and rich in proline residues. Taken together, the predicted structural features of Tpr suggest that the protein exists as an extended rod-shaped molecule with a globular highly charged C-terminus. The occasional interruptions in the coiled-coil motif indicate that Tpr may have several kinks in the rod-like domain as well. The extended shape as well as the unusual arrangement of proline residues would be expected to have a profound effect on the structure of Tpr and may account for the slower than expected electrophoretic mobility observed during SDS–PAGE.

Activation of the *met* (hepatocyte growth factor) oncogene occurs via a chromosomal rearrangement, which places *tpr* sequences from human chromosome 1 into the *met* locus on chromosome 7 [Park et al., 1986]. The result of this translocation is a chimeric gene that expresses a novel 5-kb transcript with upstream sequences from *tpr* and downstream sequences from *met*. The novel fusion protein has an N-terminus with 142 amino acids from the *tpr* gene fused to the tyrosine kinase domain of the *met* hepatocyte growth factor receptor. The extracellular and transmembrane domains as well as a portion of the cytoplasmic domain of *met* are deleted.

Tpr sequences have been found fused to the tyrosine kinase domain of the *trk*/nerve growth factor receptor in oncogenes isolated from a number of human thyroid papillary tumors [Greco et al., 1992]. An oncogenic form of the *raf* serine/threonine kinase with *tpr* sequences at its N-terminus has also been identified [King et al., 1988]. The activation of three separate protein kinases by rearrangement with the *tpr* locus suggests a specific function for the *tpr* sequences and a common mechanism of activation for these oncogenes.

One mechanism by which Tpr could activate protein kinase oncogenes is by promoting dimer formation between *tpr*/oncogene fusion proteins. This could lead to autophosphorylation and the constitutive activity of otherwise regulated protein kinases. This possibility was suggested in a report demonstrating that *tpr–met* fusion proteins are immunoprecipitated as homodimers, that the dimerization depends on the existence of at least one of the leucine zippers located at the N-terminus of Tpr, and that dimer

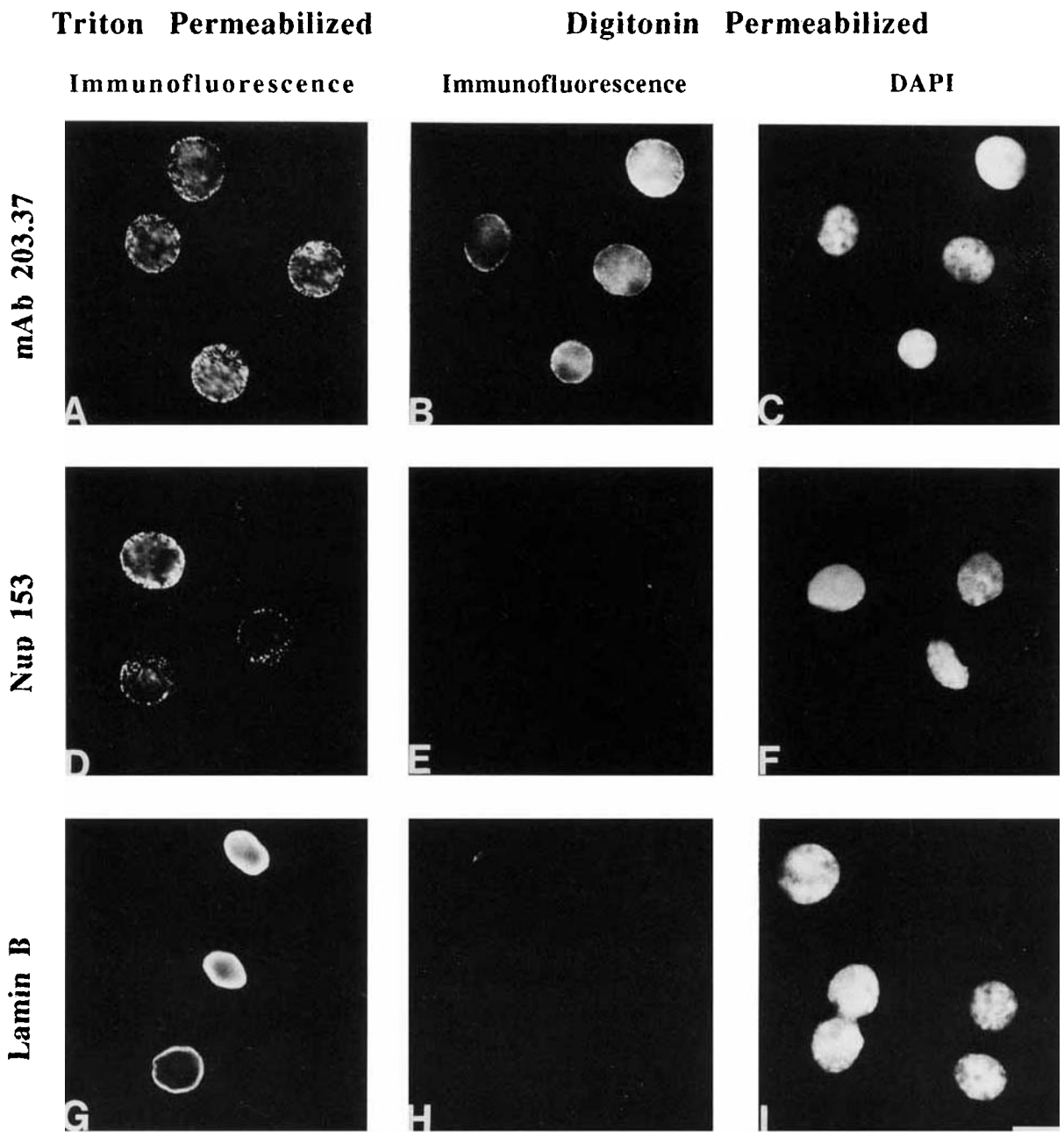

Fig. 5. Localization of Tpr to the cytoplasmic side of the nuclear pore complex. Immunofluorescent microscopy of the human carcinoma cell line ME180 permeabilized with 0.1% Triton X-100 (**A,D,G**) or 0.004% digitonin (**B,C,E,F,H,I**) prior to fixation with formaldehyde. Cells stained with mAb 203.37 show equally intense staining, whether they were permeabilized with Triton X-100 (**A**) or digitonin (**B**). **C:** Same field of cells shown in **B** stained with DAPI to show the DNA. In contrast to mAb 203.37, antibodies to the NPC protein nup153 (**D,E**) and the nuclear lamina protein lamin B (**G,H**) can only bind to their respective epitopes when the cells have been permeabilized with Triton X-100. Digitonin-permeabilized cells have intact nuclear membranes, and internal nuclear proteins are inaccessible to antibodies. **F,I:** DAPI staining of the same cells in **E** and **H**, respectively. Scale bar = 10 μm.

formation is necessary for transforming activity [Rodrigues and Park, 1993]. Further support for this hypothesis comes from the observation that the 65-kDa oncogene product $p65^{tpr-met}$ is distinct from the 140-kDa *met* hepatocyte growth factor receptor in that it is constitutively phosphorylated on tyrosine residues [Gonzatti-Haces et al., 1988].

A further possible mechanism for the transforming activity of Tpr-protein kinase fusion

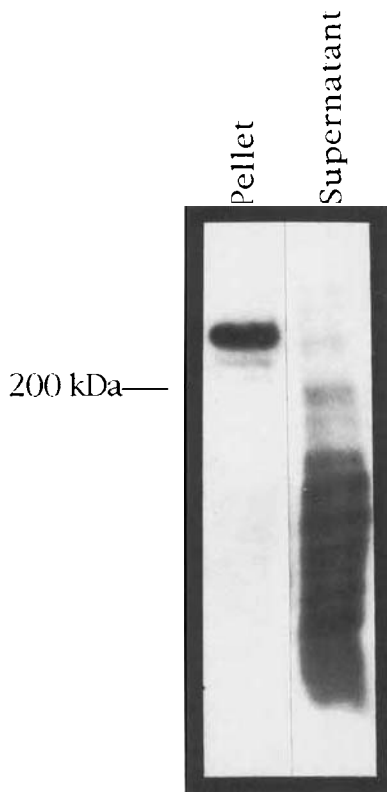

Fig. 6. Tpr is a phosphoprotein. Immunoprecipitation of Tpr from ^{32}P-orthophosphate-labeled cells. The human cervical carcinoma cell line ME180 was labeled for 12 h with ^{32}P-orthophosphate and then extracted with RIPA buffer supplemented with proteinase and phosphatase inhibitors. Tpr was precipitated from the extract with mAb 203.37 and protein G–Sepharose beads. Samples from the precipitated pellet and the supernatant were resolved by SDS–PAGE, and the gel was exposed to X-ray film.

proteins, and one that does not exclude dimerization, is that the amino acids contributed by Tpr contain a motif that directs the fusion protein to the nuclear pore complex. The localization of constitutively active protein kinases to the NPC could have a dramatic effect on the regulation of nucleocytoplasmic traffic as well as on the activity of molecules entering and leaving the nucleus. In either case, this could have a significant impact on the proliferative activity of the cell.

The disassembly and reassembly of the nuclear envelope during mitosis is regulated by cycles of phosphorylation and dephosphorylation of the nuclear lamins [Gerace and Burke, 1988]. The recent demonstration that several *Xenopus* nucleoporins are specifically phosphorylated at mitosis [Macaulay et al., 1995] raises the possibility that the assembly and disassembly of the NPC could be regulated in a fashion similar to that for the nuclear envelope. The demonstration that Tpr is also a phosphorylated component of the NPC is provocative and suggests that additional NPC components may be phosphoproteins as well. We are examining the phosphorylation state of Tpr through the cell cycle to determine whether there is a connection between phosphorylation and the assembly of the nuclear pore complex. Alternatively, phosphorylation of Tpr could play a functional role, perhaps in some aspect of regulation of nuclear transport. It would be very interesting to determine whether there exists a connection between the relative transport activity of a nuclear pore and the phosphorylation of Tpr.

The extensive coiled-coil domain that comprises the first 1,600 amino acids of Tpr suggests a structural role for this protein. A coiled-coil structure facilitates protein–protein polymerization and is frequently found in proteins involved in filament systems [Albers and Fuchs, 1992]. Examples include the cytokeratins and the nuclear lamins. The structure of Tpr suggests that it too may participate in the formation and maintenance of filamentous structures. The long coiled-coil region could promote the assembly of homo- or heteropolymers to form filaments. This, along with its position on the cytoplasmic side of the NPC, suggests that Tpr may be a part of the system of fibrils that extend from the nuclear pore into the cytoplasm. Immunoelectron microscopy has shown that rat Tpr is, in fact, localized to these cytoplasmic fibrils [Byrd et al., 1994]. In this position, Tpr could be part of the structural "backbone" of the cytoplasmic fibrils of the NPC to which other proteins, with more specific functions, are recruited. Another possibility is that Tpr may participate as a "docking" protein for macromolecules being transported into the nucleus, specifically binding to the cytoplasmic factors directly involved in the transport of macromolecules into the nucleus [Adam et al., 1989, 1990]. Further work to elucidate the function of Tpr is certainly required. One area of study that may prove helpful would be the blocking of Tpr function through the use of antibodies specific for Tpr. Antibody mediated inhibition of NPC formation or function might provide a good starting point for these investigations.

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