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Nuclear protein p68 is an RNA-dependent ATPase

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The human nuclear antigen p68 cross reacts with a monoclonal antibody to SV40 large-T antigen. Its deduced amino acid sequence contains short motifs which place it in a large superfamily of proteins of known or putative helicase activity. Recently, a p68 subfamily (DEAD box proteins) which share more extensive regions of homology has been identified in mouse, *Drosophila, Saccharomyces cerevisiae* and *Escherichia coli*. These proteins are involved in translation, ribosome assembly, mitochondrial splicing, spermatogenesis and embryogenesis. We show here that immunopurified human p68 has RNA dependent ATPase activity. In addition, we show that the protein undergoes dramatic changes in cellular location during the cell cycle.

Key words: ATPase/DEAD box/helicase/p68/T antigen

Introduction

SV40 large-T antigen is a complex multi-functional protein that is absolutely required for viral DNA replication and can transform a wide range of host cells (Rigby and Lane, 1983; Livingstone and Bradley, 1987). It interacts with key host proteins to divert them from their normal function for the benefit of the virus. It interacts directly with two anti-oncogenes, p53 (Lane and Crawford, 1979) and p105 RB (DeCaprio *et al.*, 1988), to create a cellular environment favouring viral DNA replication. It binds to the viral origin of replication (Tjian, 1978) and through its intrinsic DNA helicase activity (Stahl *et al.*, 1986) unwinds the viral template (Dean *et al.*, 1987; Wold *et al.*, 1987). It forms a complex with DNA polymerase α (Smale and Tjian, 1986; Gannon and Lane, 1987) which probably serves to introduce the polymerase to the viral origin. It binds to the transcription factor AP2 (Mitchell *et al.*, 1987) and reduces *in vitro* AP2 dependent transcription. There is strong evidence that it has dramatic effects on steady state levels of both viral and cellular transcripts (Schutzbach *et al.*, 1982; Scott *et al.*, 1983; reviewed in Acheson, 1981) although the reason for this is unclear.

There must exist cellular proteins whose normal role it is to interact with p53, p105 RB, polymerase α and AP2. It is likely that these 'T-equivalent proteins' will play an important role in cell regulation. One potential way to identify them is to look for T-related antigens in the cell. We (Lane and Hoeffler, 1980; Mole *et al.*, 1987) and others (Gurney *et al.*, 1980; Harlow *et al.*, 1981; Ball *et al.*, 1984) have generated large libraries of monoclonal antibodies against large T and used them to search for cross-reactive host proteins. One such cross reaction has been studied in detail (Lane and Hoeffler, 1980). The monoclonal antibody PAb 204 binds to a site that has been mapped with synthetic peptides to amino acids 453–469 on T antigen (Mole *et al.*, 1987). The epitope lies close to the consensus nucleotide binding motif. Antibody binding inhibits the ATPase (Clark *et al.*, 1981), DNA helicase (Stahl *et al.*, 1986) and replicative functions of large T (Smale and Tjian, 1986; Gough *et al.*, 1988). The epitope is obscured when T is bound to either DNA polymerase α or p53 and under some conditions PAb 204 may displace p53 from T (Lane and Gannon, 1986; Gannon and Lane, 1987). The antibody cross reacts very specifically with a 68 000-dalton host protein, p68, that is present in the nuclei of growing cells from a wide range of vertebrate species (Lane and Hoeffler, 1980). The antibody was used to screen λ gt11 cDNA libraries (Ford *et al.*, 1988). A cDNA was isolated whose deduced amino acid sequence is related to that of eIF4-A, a eukaryotic translation factor. Both proteins belong to a subset of the superfamily of proteins with known or suspected helicase activity described by Hodgman (1988), Lane (1988) and Gorbalenya *et al.* (1988). Recently several new members of the eIF4A subfamily have been discovered (Linder *et al.*, 1989). While genetic evidence suggests that these proteins play distinct cellular roles, their sequence homology suggests that they share a core biochemical activity as RNA helicases. We report here the isolation of p68 from human cells and demonstrate that it is is an RNA dependent ATPase. Finally, we note striking changes in the cellular location of p68 during the cell cycle.

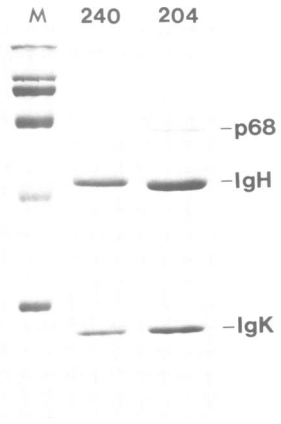

Fig. 1. SDS–polyacrylamide gel stained with Coomassie blue showing p68 immunoprecipitated from HeLa cells by monoclonal antibody PAb 204. IgH, immunoglobulin heavy chain; IgK, immunoglobulin kappa light chain; PAb 240, negative control antibody; M, relative mol. wt markers in kd: 205, 116, 97, 66, 45 and 29.

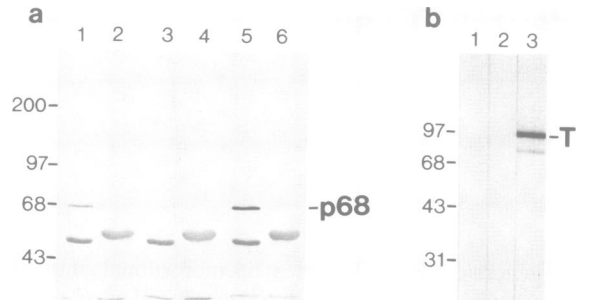

Fig. 2. (**a**) Immunoblot of immunoprecipitates from 293 cells probed with MF7 anti-p68 serum (**lanes 1** and **2**), BG2 plus normal mouse serum (**lanes 3** and **4**, negative controls) or PAb 204 (**lanes 5** and **6**). **Lanes 1, 3** and **5** contain p68 immunoprecipitated by PAb 204. **Lanes 2, 4** and **6** are BG2 immunoprecipitates (negative control). Immunoglobulin heavy and light chains are visible below the p68 band because alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin was used to detect the first antibody. (**b**) Immunoblot of SV40 large-T antigen probed with MF7 (**lane 1**), BG2 plus normal mouse serum (**lane 2**) or PAb 204 (**lane 3**). Relative mol. wt is indicated in kd.

Results

Immunopurification of p68

The p68 protein can be detected in a wide range of mammalian cells by immunoblotting with PAb 204 (data not shown). Initial attempts to purify p68 by immunoaffinity chromatography were unsuccessful because we lysed the cells at physiological salt concentration, which extracts much less p68 than 0.5 M salt (data not shown). In addition, PAb 204 is an IgG1 kappa mouse monoclonal antibody with a low affinity for protein A. The hybridoma was therefore grown in serum free medium, from which antibody could easily be purified either bound to protein G–Sepharose beads or by conventional means (Harlow and Lane, 1989). We immunoprecipitated p68 from concentrated HeLa and 293 cell extract with the antibody on protein G beads. Coomassie blue staining of HeLa material run on a polyacrylamide gel (Figure 1) reveals a single band at 68 kd which is absent from the control immunoprecipitate performed with a monoclonal antibody of identical isotype grown under identical conditions. The other two bands seen are immunoglobulin heavy and light chain.

Antisera to a p68 fusion protein confirm the identity of our human p68 cDNA clone

To confirm that our human cDNA clone (Ford *et al.*, 1988) is authentic p68 rather than another host protein which cross reacts with PAb 204, we immunized mice with the cloned p68 expressed as a fusion protein in *E.coli*. Antisera to the fusion protein detect a 68 kd band on immunoblots of authentic p68 immunoprecipitated from 293 cells (Figure 2a). Because the fusion protein contains the PAb 204 epitope, it is formally possible that the serum is directed only against this epitope. We exclude this possibility by showing that the serum does not recognize SV40 large-T antigen (Figure 2b).

p68 is an RNA dependent ATPase

p68 is homologous to known RNA and DNA dependent ATPases (Hodgman, 1988; Lane, 1988; Gorbalenya *et al.*, 1988) and antigenically related to T antigen, a DNA

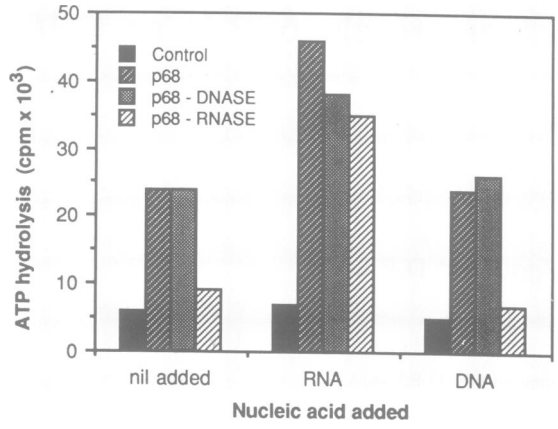

Fig. 3. ATPase assays showing that p68 is stimulated by RNA but not DNA. Immunoprecipitates with PAb 204 (p68) or PAb 240 (control) were incubated with 50 μM γ-^{32}P-labelled ATP. ATP hydrolysis was measured as release of free $^{32}P_i$ (10^3 c.p.m. is ~1 fmol/s). For details see text.

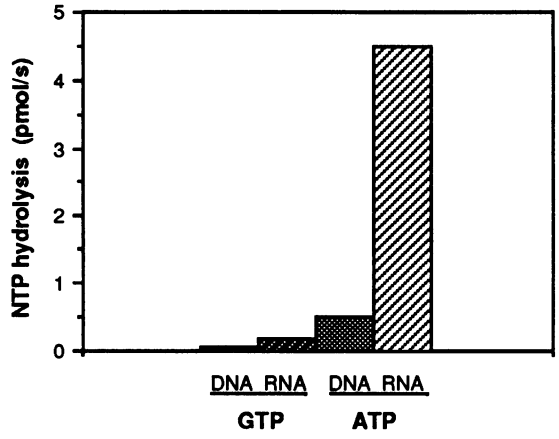

Fig. 4. ATPase and GTPase assays showing that p68 does not hydrolyse GTP. Immunoprecipitated p68 was incubated with 1 mM γ-^{32}P-labelled ATP or GTP and $^{32}P_i$ release measured in the presence of 0.2 mg/ml HeLa cell RNA or salmon testis DNA. The p68 was prepared from cells lysed in high salt and RNase A. Background counts due to spontaneous NTP hydrolysis have been deducted.

dependent ATPase. We have tested HeLa cell p68 for nucleic acid dependent ATPase activity. Because p68 is difficult to purify we chose to examine its activity while still bound to PAb 204 on protein G beads. The PAb 204 binding site on p68 lies outside the region of homology with eIF4-A and distant from the ATPase and helicase motifs in the primary sequence (Ford *et al.*, 1988). We used the assay of Clark *et al.* (1981) in which ATP hydrolysis is measured as release of $^{32}P_i$ from γ-^{32}P-labelled ATP.

When cells are lysed at physiological salt concentration, we find that immunoprecipitated p68 has substantial ATPase activity which is stimulated by added RNA but not by DNA (Figure 3). We used HeLa cell RNA and boiled sonicated salmon testis DNA (0.2 mg/ml) to stimulate the ATPase because these should contain a wide spectrum of secondary structures. The presence of significant ATPase activity even without added RNA made us consider the possibility that there was endogenous nucleic acid in our immunoprecipitates. To address this question, we pretreated immunoprecipitates with either DNase I (100 U/ml) or RNase A

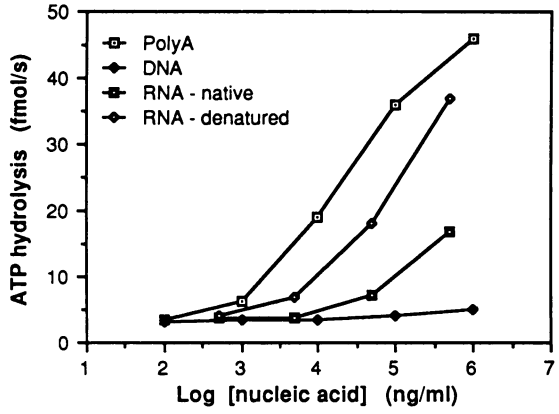

Fig. 5. ATPase assays showing that p68 is stimulated more effectively by single-stranded than double-stranded RNA. Immunoprecipitated p68 was incubated with 10 μM ATP and $^{32}P_i$ release measured in the presence of increasing amounts of poly(A), salmon testis DNA (DNA), native rotavirus genomic RNA (RNA-native) or DMSO-denatured rotavirus genomic RNA (RNA-denatured). The immunoprecipitates were RNase treated to reduce endogenous activity.

(0.2 mg/ml) and then washed them thoroughly to remove the nuclease. RNase treatment reduces ATPase activity to near control levels. Only RNA can restore activity to RNased beads. DNA has no effect, even on DNase treated p68. We conclude that with cell lysis at 150 mM NaCl there is probably endogenous RNA which co-purifies with p68.

We have obtained a *K*m value for ATP of 10^{-3} to 10^{-4} M. This is one order of magnitude higher than that of eIF4-A (Grifo *et al.*, 1984), although still below the cellular ATP level (10^{-2} to 10^{-3} M). We therefore tested p68 for GTPase activity (Figure 4). We see no GTPase activity with either RNA or DNA, which is consistent with the lack of the NKXD/W GTPase motif (Dever *et al.*, 1987) in p68.

We have not seen significant differences in ATPase activity with different species of cellular RNA or poly(A). The ATPase activity of eIF4-A is reduced by secondary structure in the RNA template (Abramson *et al.*, 1987), which is one of the hallmarks of a helicase. We therefore tested p68 with double-stranded rotavirus genomic RNA. The viral RNA was divided into equal aliquots, one of which was denatured using the dimethyl sulphoxide (DMSO) technique, which minimizes RNA degradation (McCrae and Joklik, 1978). In its double stranded form the RNA is less effective at stimulating ATP hydrolysis than it is after DMSO treatment (Figure 5).

Cell cycle variation in p68 distribution

Immunohistochemical examination of mammalian cells with PAb 204 reveals a weak granular staining pattern which is localized exclusively in the cell nucleus but spares the nucleoli. The extent of granularity depends on the individual cell type. PTK-1 cells characteristically give an exceptionally punctate pattern (Lane and Hoeffler, 1980). In mitotic cells PAb 204 does not stain chromosomes on the metaphase plate although weak peri-chromosomal staining is observed. The most striking phenomenon is the presence of a few intensely staining bodies in the nuclei of cells that have just divided. The granules usually number two or three arranged in a symmetrical pattern in sister cell nuclei (Figure 6). The

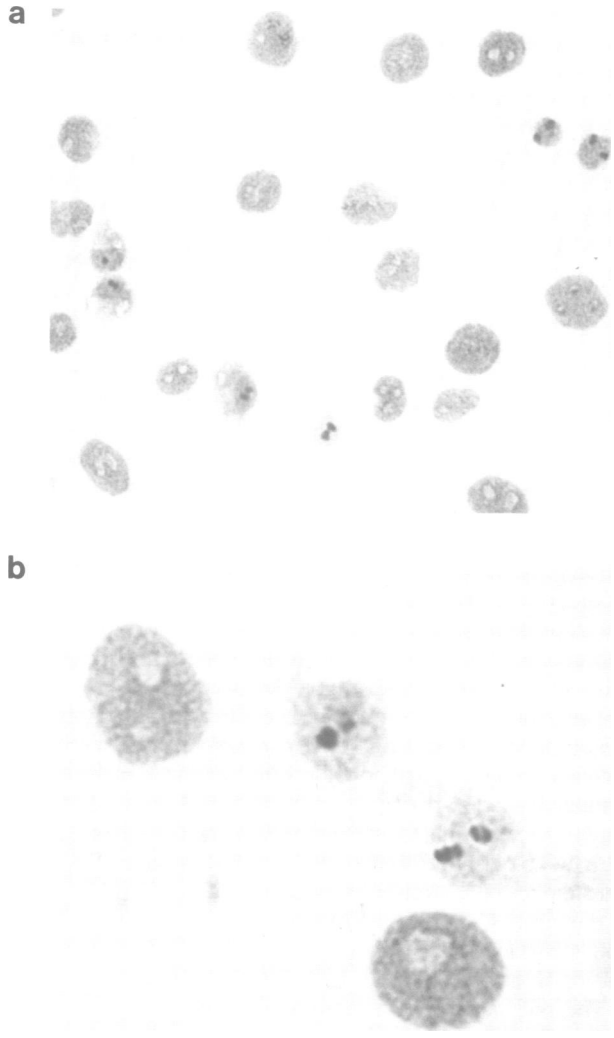

Fig. 6. Immunoperoxidase staining of p68 in CV1 cells using PAb 204, rabbit anti-mouse HRP and diaminobenzidine. (**a**) Low power, (**b**) high power.

pattern is present in all the cell lines we have examined to date.

Discussion

When we first reported the homology between p68 and eIF4-A we speculated that it might define a new gene family. The recent identification of four diverse new genes related to p68 confirms this idea. Linder *et al.* (1989) proposed the term 'DEAD box' proteins to describe the family, because the sequence aspartate, glutamate, alanine, aspartate (part of the ATP binding site) uniquely identifies these proteins in the database. The sequence data strongly suggests that they share a core biochemical activity.

We report here that p68 is an RNA dependent ATPase which is highly stimulated by a range of natural and synthetic RNAs. It is stimulated weakly by dsRNA and not at all by DNA. Two lines of evidence lead us to suspect that p68 is bound to nuclear RNA: first, p68 extracted in conditions which favour RNA–protein interactions has endogenous ATPase activity which can be abolished by RNAse treat-

ment; and second, the PAb 204 cell staining pattern is abolished by RNase A treatment of fixed cells (data not shown).

Three DEAD box proteins (eIF-4A, Grifo *et al.*, 1984; srmB, Nishi *et al.*, 1988; and p68) have now been shown to be RNA dependent ATPases and it is reasonable to assume that all members of the family possess this activity. We speculate that these proteins bring the power to manipulate RNA secondary structure to many different molecular organelles in the cell. Only eIF4-A has a clearly defined role: by melting secondary structure near the mRNA cap it allows the ribosome to scan along the message looking for the start codon. Cap dependence requires that eIF4-A interact with several other initiation factors (reviewed in Sonenberg, 1988). The strong inference for other members of the family is that they will be subunits of multimeric complexes that act on specific RNAs. For example, MSS116 (Seraphin *et al.*, 1989) is probably a component of the mitochondrial splicing apparatus, which presumably requires the ability to modify secondary structure in pre-mRNA. Vasa protein is a component of *Drosophila* polar granules, complex ribonucleoprotein particles which are thought to govern the expression of some posterior group maternal effect genes (Hay *et al.*, 1988; Lasko and Ashburner, 1988).

This concept may hold the key to understanding the biological significance of the cross reaction between p68 and SV40 T antigen. T is known to bind, in part covalently, to cellular RNA (Darlix *et al.*, 1984; Carroll *et al.*, 1988). It may replace p68 in ribonucleoprotein particles by interacting with p68's normal ligand via the PAb204 epitope. T antigen thus mimics p68 in order to recruit the particle for the benefit of the virus. Conservation of the PAb 204 epitope on T and p68 is thus analogous to conservation of the p105 RB binding site on T and adenovirus E1A (Moran, 1988).

The p68 protein, like many other nuclear antigens, has a granular distribution in the interphase cell nucleus and appears to be excluded from the nucleoli. Granular staining is associated with a variety of functional entities including sites of DNA replication (Bravo and Macdonald-Bravo, 1987) and ribonucleoprotein particles (Spector *et al.*, 1987). The antigen is either absent or inaccessible to PAb 204 on condensed chromosomes but as sister cells emerge from mitosis the antigen is strikingly clustered. At this stage in the cell cycle nucleoli are being assembled at the chromosomal nucleolar organizers and it is possible that the protein may be associated with the formation of nucleoli. Interestingly another DEAD box protein, srmB (Nishi *et al.*, 1988), appears to be involved in ribosome assembly. Previous studies on mitosis (Albrecht-Buehler, 1977) drew attention to mirror symmetry in post-mitotic sister cells. The results with p68 extend this observation to the cell nucleus and imply that despite complete disruption at mitosis, elements of nuclear architecture are recalled after cell division.

Materials and methods

Cell lines, reagents and buffers

Mouse monoclonal hybridomas PAb 204 (Lane and Hoeffler, 1980), PAb 240 and BG2 were produced in this laboratory. CV1 cells were provided by ICRF cell production. 293 cells were provided by Dr P.Gallimore.

Protein G–Sepharose was purchased from Pharmacia. RNase-free DNase I was purchased from Stratagene. Rabbit anti-mouse immunoglobulins conjugated to horseradish peroxidase (HRP), rabbit anti-mouse immunoglobulins conjugated to alkaline phosphatase (ALP) and normal mouse serum were purchased from Dako. Serum free hybridoma culture medium (HB101) was purchased from Dupont. Rotavirus genomic RNA was kindly provided by Dr Malcolm McCrae. Total cellular RNA was made from HeLa cells by the guanidinium method (Ausubel *et al.*, 1987). SV40 large-T antigen was prepared by the method of Simanis and Lane (1985). ^{32}P-labelled ATP and GTP were purchased from Amersham. Unless stated otherwise all other reagents were purchased from Sigma.

Lysis buffer is 150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 1% Nonidet P-40 (NP-40), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF). ATPase buffer is 150 mM NaCl, 5 mM KCl, 1.5 mM $MgCl_2$, 20 mM Hepes pH 7.0, 1 mM DTT, 1 mM PMSF. DNase buffer is 150 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$, 20 mM Pipes pH 7.0, 1 mM DTT.

Immunoprecipitation

3×10^9 HeLa or 293 cells were incubated in lysis buffer for 30 min on ice. The cell extract was centrifuged at 100 000 *g* for 30 min and the pellet discarded. Monoclonal antibody was added either in solution (Figures 1, 2 and 3) or pre-bound to protein G–Sepharose beads (Figures 4 and 5) and incubated on a rotating wheel overnight at 4°C. Protein G–Sepharose beads were added (Figures 1, 2 and 3) and the incubation continued for 1 h. The beads were then washed in lysis buffer.

The lysis conditions were varied as follows in individual experiments: 500 mM NaCl and sonication (Figures 4 and 5); RNase A 0.1 mg/ml (Figures 1, 4 and 5). After washing, the beads for Figure 5 were incubated in lysis buffer containing 0.1 mg/ml RNase A for 1 h. Finally, the beads were washed in ATPase buffer (Figures 1, 2, 4 and 5), or DNase buffer (Figure 3). Subsequent manipulations are described in the text.

Electrophoresis and immunoblotting

Denaturing polyacrylamide gel electrophoresis and immunoblotting were performed as described in Harlow and Lane (1989) using BioRad minigel apparatus. Protein from 10 μl of beads immunoprecipitated as above was loaded per track (Figures 1 and 2a). Pure T antigen was loaded in a single slot, blotted and strips cut from the blot with ~10 ng of T antigen per strip. Blots were blocked in 2% dried milk in phosphate buffered saline (PBS) and washed in 1% NP-40 in PBS. Antibodies were suspended in blocking buffer plus 0.1% Tween: PAb 204 and BG2 diluted 1:2, MF7, normal mouse serum and rabbit anti-mouse ALP diluted 1:1000. Blots were developed with bromo-chloro-indolyl phosphate and nitro blue tetrazolium. MF7 is pooled serum from mice immunized with a β-galactosidase fusion protein made in *E.coli* by expressing a PUR290 plasmid with the p68 cDNA (Ford *et al.*, 1988) cloned into the *Eco*RI site. The fusion protein was purified as described in Harlow and Lane (1989).

ATPase assays

ATPase and GTPase activity is expressed as P_i released from γ-^{32}P-labelled ATP or GTP. Assays were performed in 30 μl ATPase buffer containing ~ 10 μl of protein G–Sepharose beads bound to PAb 204 or PAb 240. From Coomassie staining of polyacrylamide gels we estimate that there was ~50 ng p68 per assay. Nucleic acid and nucleotide triphosphate were included as indicated in the text. The assays were incubated for 30 min at 37°C. 300 μl of 7% activated charcoal in 50 mM HCl, 5 mM H_3PO_4 was then added and the mixture centrifuged at 10 000 *g* for 10 min to remove unreacted ATP. The supernatant (containing free $^{32}P_i$) was mixed with Aquasol liquid scintillation cocktail and counted in a Packard scintillation counter on the ^{32}P channel.

Cell staining

CV1 cells grown on cover slips in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) were fixed in cold 50% acetone in methanol for 5 min and dried. They were then incubated for 3 h with PAb 204 hybridoma supernatant, rinsed in PBS, incubated for 3 h with rabbit anti-mouse HRP diluted 1:100 in 10% FBS in PBS, rinsed in PBS and developed with diaminobenzidine.

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