Pathological mutations in TSC1 and TSC2 disrupt the interaction between hamartin and tuberin

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Critical functions of hamartin and tuberin, encoded by the TSC1 and TSC2 genes, are likely to be closely linked. The proteins interact directly with one another and mutations affecting either gene result in the tuberous sclerosis phenotype. However, the regions of hamartin and tuberin that interact have not been well defined, and the relationship between their interaction and the pathogenesis of tuberous sclerosis has not been explored. To address these issues a series of hamartin and tuberin constructs were used to assay for interaction in the yeast two-hybrid system. Hamartin (amino acids 302–430) and tuberin (amino acids 1–418) interacted strongly with one another. A region of tuberin encoding a putative coiled-coil (amino acids 346–371) was necessary but not sufficient to mediate the interaction with hamartin, as more N-terminal residues were also required. A region of hamartin (amino acids 719–998) predicted to encode coiled-coils was capable of oligomerization but was not important for the interaction with tuberin. Subtle, non-truncating mutations identified in patients with tuberous sclerosis and located within the putative binding regions of hamartin (N198_F199delinsI;593–595delACT) or tuberin (G294E and I365del), abolished or dramatically reduced interaction of the proteins as assessed by yeast two-hybrid assays and by co-immunoprecipitation (G294E and I365del). A region of hamartin (amino acids 302–430) and tuberin (amino acids 1–418) interacted strongly with one another. A region of tuberin encoding a putative coiled-coil (amino acids 346–371) was necessary but not sufficient to mediate the interaction with hamartin, as more N-terminal residues were also required.

INTRODUCTION

TSC1 and TSC2 are tumour suppressor genes that were originally identified through their involvement in the inherited disorder tuberous sclerosis (TSC) (1,2). They encode the previously unknown proteins hamartin and tuberin. Studies of the hamartomatous growths that characterize TSC have revealed inactivation of either hamartin or tuberin by a classical two-hit mechanism (2–6). Comparable findings have also been reported in lesions, particularly renal adenomas and carcinomas, from naturally occurring and engineered mutant Tsc1 and Tsc2 rodent models (7–12).

Abnormalities of cellular proliferation, differentiation and migration have been indicated by examination of various TSC lesions, suggesting that hamartin and tuberin are required for the normal regulation of these cellular processes (13). In human, rodent and Drosophila cells, overexpression of hamartin and tuberin lengthens the G1 phase of the cell cycle and is associated with an inhibition of cell proliferation and, in Drosophila, reduction in cell size. Conversely, deficiency of hamartin or tuberin in null cells derived from rocky (rcy) (Tsc1 –/–) and gigas (gig) (Tsc2 –/–) Drosophila mutants or the Eker (Tsc2 –/–) rat is associated with shortening of G1 and, in Drosophila, with hypertrophy and normal ploidy (14–19). Giant cells with normal DNA content are also a characteristic feature of central nervous system hamartomas in TSC (20). It is currently unclear whether hamartin and tuberin affect the cell cycle directly or indirectly or exactly how these effects relate to the pathogenesis of TSC. Recent reports suggest that the proteins act downstream of the insulin receptor, PTEN and Akt proteins (19) to influence dS6K, cyclins A, B, D and E (15,18,19,21) and p27 (16).

The C-termini of hamartin (amino acids 719–998), that includes putative coiled-coil domains (2), and of tuberin (amino acids 1049–1809), that includes a GTPase activating protein (GAP)-related domain (1) with reported activity for the Ras/Rho and Ral GTPases (22,23) appear important for growth suppression (14,17). Ezrin binding (amino acids 881–1084) and Rho activation (amino acids 145–510) by hamartin were recently assessed to be important in the assembly of contractile actomyosin filaments with downstream effects on the cytoskeleton and cell-substrate adhesion (24). Such roles could underlie the aberrant cell migration that characterizes cerebral pathology in TSC patients (25), and the apparent spread of abnormal smooth muscle cells from renal cell angiomylipomas to the lung in TSC-associated lymphangioleiomyomatosis (26).

Whereas distinct functions have been postulated for different regions of hamartin and tuberin, evidence linking these functions to the interaction between the proteins and to the TSC phenotype has been lacking. In contrast, recent evidence

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suggests that the *Drosophila rcy/Tsc1* and *gig/Tsc2* phenotypes result from disruption to the hamartin–tuberin complex (18,19). Many co-immunoprecipitation studies have demonstrated a physical interaction between full-length hamartin and full-length tuberin (19,27–32). The complex has been detected in all phases of the cell cycle (17) and appears to stabilize tuberin by preventing its ubiquitination and its subsequent degradation (33). In this study we sought to clarify the areas in hamartin and tuberin that are important for their interaction, and to study the effects of TSC-associated mutations on their binding.

**RESULTS**

Full-length hamartin and tuberin were shown to co-immunoprecipitate from lysates of transfected Cos7 cells overexpressing both proteins, consistent with previous reports of a direct interaction between the proteins (19,27–32) (Fig. 1). A series of tuberin constructs representing progressive truncations from the C-terminal end of tuberin were assayed for interaction with a large fragment of hamartin encompassing residues 335–1161 by yeast two-hybrid assay (Fig. 2). A construct corresponding to tuberin residues 1–418 gave the largest number of colonies on selection for histidine auxotrophy, and the most intense β-galactosidase activity, indicating the strongest interaction (Fig. 2A). This region of tuberin includes a weakly predicted coiled-coil structure at amino acids 340–400 that was previously reported to mediate tuberin’s interaction with hamartin (27). However, constructs corresponding to tuberin residues 337–415, 270–415 and a region N-terminal to residue 270 did not interact with hamartin 335–1161, indicating that neither the weakly predicted coiled-coil region nor the more N-terminal region alone could mediate the interaction and that both were required. Larger tuberin constructs that, in addition, contained more C-terminal sequences interacted more weakly than the amino acids 1–418 tuberin construct (Fig. 2A).

In yeast two-hybrid assays using a range of hamartin constructs, all of those that included part of the region corresponding to residues 171–430 interacted with full-length tuberin and with its N-terminal region (residues 1–418) (Fig. 2B). In assays using the full-range of hamartin and tuberin constructs, the strongest interactions were seen between those encoding residues 335–430 of hamartin and 1–418 of tuberin. An interaction was also seen, although more weakly, with N-terminal constructs of hamartin, suggesting the presence of multiple binding sites within its first 430 residues. In contrast, a series of C-terminal hamartin constructs that included part or all of the predicted coiled-coil regions (amino acids 719–998) (2) interacted very weakly or not at all with full-length and truncated tuberin constructs (Fig. 2B).

Next, we investigated the effects on hamartin–tuberin binding of well characterized small in-frame deletions and a missense mutation that have been reported in patients with TSC, and that map to the areas that appeared important for interaction. Introduction of either the missense change G294E (34) or the 3 bp deletion I365del (34) into tuberin abolished the ability of tuberin 1–418 to bind to hamartin in yeast two-hybrid assays (Fig. 2A). In contrast, three non-pathogenic amino acid substitutions, R261W, M286V and R367Q (34–36) that flanked the *TSC2* mutations had no or little effect on the interaction with hamartin (Fig. 2A). *TSC1* mutations that do not lead to protein truncation are extremely rare and we have been able to verify only one example. We found that this well characterized mutation (N198_F199delinsI;593–595delACT) (37) abolished the interaction of hamartin with tuberin in the yeast two-hybrid assay (Fig. 2B).

Next, we investigated the effects of the TSC associated mutations and the non-pathogenic missense variants on the ability of hamartin and tuberin to co-immunoprecipitate in lysates from Cos7 cells. As we had observed for the wild-type proteins, full-length tuberin that contained each of the three non-pathogenic missense variants co-immunoprecipitated with full-length hamartin (Fig. 3). In contrast, full-length tuberin containing either the G294E or the I365del 3 bp deletion mutation, although stable and overexpressed at the same level as the wild-type protein, failed to co-immunoprecipitate with hamartin (Fig. 3), confirming that these pathological TSC2 mutations disrupt the ability of tuberin to bind to hamartin. The *TSC1* 3 bp deletion mutation (N198_F199delinsI;593–595delACT) (37), that lies upstream of the predicted minimal binding...
region for tuberin, was introduced into full-length hamartin. Although the mutant protein was shown to be expressed at a similar level to wild-type there was a marked reduction in its ability to immunoprecipitate tuberin (Fig. 3B).

**DISCUSSION**

Both TSC1 and TSC2 are considered to act as tumour suppressor genes, since inactivation of the corresponding wild-type allele through allelic loss or intragenic mutation has been reported in a wide variety of hamartomas from patients with TSC (5,38). Studies of lesions from the spontaneous Eker (Tsc2+/−) rat model and from engineered Tsc1 and Tsc2 ‘knockout’ mice (7,9,11,12,39), together with phenotypic rescue of the Eker rat by a Tsc2 transgene (40) support this conclusion. In mammalian cell culture systems, reduction of cellular proliferation by hamartin or tuberin has been associated with lengthening of G0/G1 (15–17). Similar cell cycle effects have been reported in Drosophila, where growth was also restricted as a result of cell size reduction (18,19). These

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*Figure 2. Hamartin–tuberin interaction assessed by yeast two-hybrid assay. (A) Tuberin constructs versus hamartin amino acids 335–1161. A series of tuberin constructs representing progressive truncation from the C-terminus were assayed for interaction with hamartin. Tuberin constructs were fused to the GAL4 transactivation domain or binding domain and assayed against hamartin cloned into the complimentary GAL4 vector. Top, a cartoon represents full-length tuberin with selected putative structural and functional domains indicated above as grey squares (LZ, leucine zipper; CC, potential coiled-coil domains; GAP, GTPase activating protein-related region). The positions of pathogenic TSC2 mutations that were also assayed are indicated as a red asterisk (G294E) and a red triangle (I365del) and the positions of the assayed non-pathogenic amino acid substitutions are indicated below (filled black circles). Represented below this are the tuberin constructs that were cloned into GAL4 activating and binding domain vectors. Colour coding (see interaction key) indicates the relative strength of their binding to hamartin as assessed by hisitidine auxotrophy and intensity of β-galactosidase activity. Strongest binding was observed with a construct representing amino acids 1–418 of tuberin. Below are shown the effects of the pathogenic mutations and non-pathogenic amino acid substitutions on binding between the amino acids 1–418 tuberin construct and hamartin amino acids 335–1161. Bottom, shows representative colony growth on -L-T plates (selecting for transformants containing both activating and binding domain plasmids) and on -H-L-T plates (selecting for protein interaction) and representative interaction dependent β-galactosidase activity resulting in a blue colour in colony lift assays. Assessment of interaction was on a four-point scale (+++, ++, + or –) based on the number of colonies capable of growth on selection for histidine auxotrophy (-H), and intensity of blue colour as a measure of β-galactosidase activity. Numbers in red relate the results in the Top to the Bottom. (B) Hamartin constructs versus tuberin amino acids 1–418. A series of hamartin constructs were assayed for interaction with tuberin. Top, the cartoon represents full-length hamartin with putative coiled coil (CC) regions indicated (grey squares) and the position of the assayed pathological mutation N198_F199delinsI;593–595delACT (red triangles). Strong binding was seen with a construct representing hamartin amino acids 335–430 and with all larger constructs spanning this region. Shown below this are the effects on binding of the non-truncating TSC1 mutation N198_F199delinsI;593–595delACT that lies upstream of the putative minimal binding region. Bottom, growth of yeast colonies on -L-T and -H-L-T plates with selected wild-type and mutant (TSC1 N198_F199delinsI;593–595delACT). Assessment of interaction was on a four-point scale (+++, ++, + or –) based on the number of colonies capable of growth on selection for histidine auxotrophy (-H), and intensity of blue colour as a measure of β-galactosidase activity. Numbers in red relate results from the Top panel.*
effects in Drosophila were only seen when both proteins were overexpressed together, suggesting an interdependency of hamartin and tuberin for their growth regulatory functions (19). In contrast, an anti-proliferative effect of each of the proteins has been reported on independent overexpression in mammalian cell culture (15–17). Previous studies have even indicated that overexpression of only the C-terminal regions of hamartin (residues 788–1153) or tuberin (1049–1809) can mediate growth suppression in this setting (14,17). We sought to clarify whether at least some TSC disease-related functions of human hamartin and tuberin require a direct interaction between the proteins. Our data, showing that certain non-truncating yet TSC causing mutations of hamartin and tuberin disrupt their binding supports this proposition, whether or not the binding dependent functions are among those already suggested by in vitro studies. Each of the mutations investigated was selected because it was well characterized and mapped to the regions implicated in hamartin–tuberin binding by our yeast two-hybrid experiments. Immunoblotting indicated that all were associated with production of stable mutant forms of hamartin or tuberin. Their dramatic effects on binding would be consistent with disruption of the interacting regions. In contrast, TSC causing missense mutations of tuberin that map outside the interacting region (N1643K, N1651S, N1681K) encode stable proteins that can still bind hamartin (32; unpublished data). We conclude that the binding of hamartin and tuberin is necessary, but not sufficient for their mediation of growth suppression.

Previous work has not assessed in detail the regions of hamartin and tuberin that mediate their interaction. The results of two-hybrid experiments reported here are consistent only in part with the single previous report of hamartin–tuberin binding in the yeast two-hybrid system (27). That report suggested the region encoded by amino acids 346–371 of tuberin was required for interaction with hamartin. Our data indicated that the interaction requires a more extended N-terminal region of tuberin. In contrast to the previous report (27), we did not find that the N-terminal of tuberin interacted with the C-terminal coiled-coils region of hamartin (amino acids 786–1010). We found no interaction or only very weak interaction with a large set of hamartin constructs from this region. Neither does the disruption of tuberin binding that we observed in association with the N-terminal N198_F199delinsI;593–595delACT mutation of hamartin support mediation of the interaction via hamartin’s C-terminal coiled-coils region. Rather, our data indicate that interaction of the proteins occurs primarily through their N-terminal domains.

Although tuberin 1–418 interacted strongly with hamartin, larger tuberin constructs that included the N-terminal binding region interacted much more weakly. This could reflect inappropriate folding of some truncated tuberin fragments or the masking of the binding site in progressively larger fragments. Unmasking of the N-terminal binding site to protein partners is required by the ERM proteins ezrin, radixin and moesin and related family member merlin isoform 1, for activity (41–43). It is also possible that the physiological interaction between hamartin and tuberin is complex and that assays in the yeast nucleus do not fully reflect this. A recent report showed that phosphorylation of tuberin that was dependent on specific C-terminal residues, could regulate its binding to hamartin (32). In light of our data this suggests that

Figure 3. Pathological mutations of TSC1 and TSC2 abrogate co-immunoprecipitation of hamartin and tuberin. (A) Top, Cos7 cells were transfected with full-length wild-type hamartin and FLAG-tagged full-length tuberin constructs. Lysates were immunoprecipitated using anti-FLAG antibody and immunoblotted with anti-hamartin antibody. Lane 1, wild-type tuberin; lane 2, pathogenic tuberin G294E; lane 3, pathogenic tuberin I365del; lane 4, non-pathogenic tuberin missense variant R261W; lane 5, non-pathogenic tuberin missense variant M286V; lane 6, non-pathogenic tuberin missense variant R367Q; C, empty plasmid control. Centre and bottom, immunoblotting of pre-immunoprecipitation lysate showing expression of hamartin and of wild-type and mutant forms of tuberin. (B) Top, Cos7 cells were transfected with full-length wild-type tuberin and FLAG-tagged full-length hamartin constructs. Lysates were immunoprecipitated using anti-FLAG antibody and incubated with anti-tuberin antibody. Lane 8, wild-type hamartin; lane 9, mutant hamartin N198_F199delinsI;593–595delACT; C, empty plasmid control. Centre and bottom, immunoblotting of pre-immunoprecipitation lysate showing expression of tuberin and of wild-type and mutant forms of hamartin.
critical functions of hamartin and tuberin themselves could be regulated through changes in binding.

The generation of similar or identical phenotypes through mutations at two or more loci (locus heterogeneity) is well established in many mendelian disorders. It may reflect disruption of different steps in a common pathway or equivalent effects resulting from disruption of distinct pathways as well as alterations in the different components of a functional complex. An example of the latter situation is hereditary non-polyposis colorectal cancer, that is associated with mutations in one or other of the $hMLH1$, $hMSH2$, $hMSH6$, $hpMS2$ or $hMLH3$ genes. Their products form several multimeric complexes involved in the repair of distinct classes of base pair mismatches (44). However, to our knowledge, non-truncating mutations that prevent complex formation rather than abrogate the functions of the component proteins have not been clearly documented in tumour predisposition syndromes. In contrast, molecular mechanisms analogous to that we propose for some cases of TSC are well known in inherited metabolic disease. For example, certain missense mutations of either the LDL receptor gene or the apoB-100 gene that encodes its ligand both lead to the phenotype of hypercholesterolaemia by impairing binding between the gene products (45,46). The likelihood that some cases of TSC arise through disruption of the hamartin–tuberin interaction may raise opportunities for intervention. Although it is difficult to envisage effective delivery of either protein to all deficient cells, the identification of small molecules that could act as a surrogate for hamartin’s binding to tuberin may be more tractable.

MATERIALS AND METHODS

**TSC1 and TSC2 constructs**

In-frame hamartin and tuberin constructs were made in the yeast two-hybrid activating domain vectors pGAD424 series, pGAD GH (Clontech, Basingstoke, UK) and pAD GAL4-2.1 (Stratagene, Amsterdam, The Netherlands) and in the binding domain vectors pGBK9 (Clontech), pBD-GAL4-Cam (Stratagene), pAS1-CY2 (Clontech) and pYTH9 (a gift from Richard Lamb). For expression and co-immunoprecipitation studies constructs were produced in the mammalian expression vectors pCMV-Tag2 series (for detection with an anti-FLAG antibody) and pCMV-Tag3 series (for detection with an anti-c-Myc antibody) (Stratagene).


The description of changes (mutations and polymorphisms) at the DNA and protein level are according to the nomenclature described by Antonarakis (47) and den Dunnen and Antonarakis (48). TSC-associated missense changes and small in-frame deletion mutations were created by either cloning RT–PCR products from patient-derived EBV-transformed lymphoblastoid cells into the appropriate vector and screening for the mutant allele by sequencing or by site-directed mutagenesis using the Quik Change kit, according to the manufacturer’s protocol (Stratagene). The TSC-associated mutations investigated were G294E, in exon 9 of TSC2 (34), the TSC2 in-frame deletion mutation I365del in exon 10 of TSC2 (34), and the TSC1 mutation N198_F199delinsL593–595delACT that has been shown to arise de novo in a sporadic TSC case with subsequent transmission to that individual’s two affected offspring (37). Three amino acid changing polymorphisms that we and others have identified previously (34–36) in the same region as the pathogenic TSC2 mutations were also created by site-directed mutagenesis using the Quik Change kit, according to the manufacturer’s protocol. These were: R261W, 781C→T; M286V, 856A→G and R367Q, 1100G→A.

**Yeast two-hybrid interactions**

Yeast host strain CG-1945 was sequentially transformed with 1 µg of each plasmid according to the method specified in the Clontech MATCHMAKER two-hybrid protocol (Clontech). Briefly, for primary transformation of the first construct a single yeast colony was grown in 20 ml of YPD broth (2% peptone, 1% yeast extract, 2% glucose) for ∼24 h at 30°C with shaking at 250 r.p.m. (Beckman Innova 4230 incubator), to an OD600 of 1–2. This culture was transferred to 300 ml YPD broth and grown for a further 3 h. After pelleting the cells at 1000 g for 5 min at room temperature, the pellet was resuspended in 50 ml sterile water and re-pelleted. After removing the supernatant, the cells were resuspended in 1.5 ml of 0.1 M lithium acetate/TE buffer (10 mM Tris, 1 mM EDTA pH 7.5). 100 µl of resuspended cells were added to 1 µg of each construct to be transformed, along with 100 µg of salmon sperm carrier DNA (Stratagene). To this was added 600 µl of a mix of 40% polyethylene glycol 3350 (Sigma, Poole, UK), 0.1 M lithium acetate/TE buffer. The mixture was incubated at 30°C with shaking (200 r.p.m.) for 30 minutes. After adding 10% dimethyl sulfoxide (Sigma), the cells were heat-shocked for 15 min at 42°C. The cells were briefly chilled on ice and pelleted for 1 min at 10 000 g and resuspended in 100 µl TE buffer. Cells were plated on minimal media (yeast nitrogen base without amino acids) (Becton Dickinson, Oxford, UK) supplemented with an amino acid dropout mixture (Clontech manual) lacking either the amino acid leucine (-L) for selection of activating domain plasmids or tryptophan (-T) for selection of binding domain plasmids, and 2% glucose. The yeast were grown for 3–5 days at 30°C until single colonies appeared.

For secondary transformation, a single colony from the primary transformed colony was grown in 20 ml of YPD broth for 24 h with shaking until single colonies appeared. The cells were briefly chilled on ice and pelleted for 1 min at 10 000 g and resuspended in 200 µl TE buffer. Cells were plated on minimal media lacking leucine and tryptophan (-L,-T) (to indicate transformation efficiency) and minimal media lacking histidine, leucine and tryptophan (-H,-L,-T) (to indicate a
positive interaction) and grown for 3–5 days at 30°C. Colonies capable of growth on -H,-L,-T media were tested for LacZ expression by assaying β-galactosidase activity following colony lifts on to nitrocellulose filters. Filters were immersed in liquid nitrogen for 10 s followed by incubation overnight in 1.8 ml of Z buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 0.1 mM MgSO₄, pH 7) containing 2.7 µl/ml β-mercaptoethanol and 0.334 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside in N,N-dimethylformamide (X-gal). Where possible, constructs were made in both activating and binding domain vectors and interactions were assessed using both vector combinations in sequential transformations. All experiments were repeated at least twice. Assessment of interaction was on a four-point scale (++++, ++, + or –) based on the number of colonies capable of growth on selection for histidine auxotrophy (-H), and intensity of blue colour as a measure of β-galactosidase activity.

Co-immunoprecipitation:
Full-length hamartin and tuberin were detected using a polyclonal rabbit anti-hamartin antibody raised against human hamartin residues 748–957 (33) and a rabbit anti-tuberin polyclonal rabbit anti-hamartin antibody raised against human hamartin residues 1387–1784 (KPL, St Louis, MO) or a mouse anti-c-Myc (9E10) monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively.

Cell culture and transfection:
Cos7 cells were grown in Dulbecco’s modified Eagle medium (D-MEM) with antibiotics and 10% (v/v) fetal bovine serum. All Cos7 cell co-transfections were carried out with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions, using 2 × 10⁶ cells/100 mm plate and 10 µg of DNA (5 µg each of TSC1 and TSC2 constructs).

Cell lysis, immunoprecipitation and immunoblotting:
Cells were lysed after 24 h of transfection in C-type lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 10% glycerol, 1% Triton X-100) with Complete™ protease buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1% Triton X-100) with Complete™ protease inhibitors (Roche Diagnostics, Indianapolis, IN). Lysates were clarified by microcentrifugation at 10 000 g for 10 min at 4°C, and the protein concentration of an aliquot from each lysate was determined using the bicinchoninic acid assay (Pierce, Rockford, IL). Portions of each lysate containing 300 µg protein were subjected to immunoprecipitation with 25 µl of anti-FLAG M2-agarose affinity gel or 25 µl of anti-c-Myc (9E10) monoclonal antibody-conjugated agarose, as appropriate, for 2 h at 4°C. The immunoprecipitates were washed four times with lysis buffer and four times with wash buffer (PBS pH 7.5, 5 mM MgCl₂, 1% NP-40), analysed on a 12% polyacrylamide gel, and transferred to a PVDF membrane. For all immunoblotting, the membranes were blocked overnight with milk blocking solution (KPL, Gaithersburg, MD) at 4°C, and then incubated with a 1:1000 dilution of crude antiseraum or 1 µg/ml purified antibody in KPL diluent solution for 90 min at room temperature. The membranes were washed three times for 5 min each with KPL wash solution before being incubated with a 1:5000 dilution of HRP-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ). Detection of bound antibodies was carried out using the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

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