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Heat shock protein 90 in retinal ganglion cells: Association with axonally transported proteins

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Abstract

The mRNAs for heat shock protein 90 (HSP90) are found at highest levels (differentially expressed) in the primate retinal fovea, the region of highest visual acuity, compared to the peripheral retina. HSP90 expression and retinal associations were analyzed by immuno-localization, *in situ* hybridization, and western analysis. Retinal ganglion cells (RGCs) express much of the HSP90 mRNA present in the primate retinal fovea. A large fraction of RGC synthesized HSP90 is apparently present in the axonal compartment. To identify the role of HSP90 protein in the optic nerve and retina, co-immunoprecipitation experiments were performed, using antibodies specific for HSP90 isoforms. The immunoprecipitates were analyzed for neurotrophin receptor and ligand activities, and MAP kinase activity. MAP kinase assay was used to determine the activation state of MAP kinase associated with HSP90. HSP90 proteins selectively associate with the inactive form of full-length tyrosine kinase growth factor receptor trkB, suggesting utilization during anterograde axonal transport. Activated MAP kinase, associated with the trk downstream signaling cascade, was found to co-immunoprecipitate with optic nerve HSP90, suggesting that HSP90 may be utilized in retrograde transport of the secondary messengers associated with neurotrophin signaling. HSP90 can thus be hypothesized to play a role in bidirectional RGC axonal protein transport.

Keywords: Retinal ganglion cell, trkB, Growth factor receptors, Axon transport, MAP kinase

Introduction

The heat shock protein 90 (HSP90) gene family codes for an evolutionarily conserved group of intracellular chaperone proteins central to protein-specific intracellular transport in nonneuronal systems (for review, see Csermely et al., 1998). Two HSP90 protein isoforms exist in vertebrates: HSP90β (89 kDa) and HSP90α (85kDa) (Morimoto et al., 1994). HSP90 isoforms associate *in vivo* as homodimers or heterodimers (Perdew et al., 1993). Both HSP90 mRNA isoforms are constitutively expressed, but variably induced during stress (Hansen et al., 1991; Kawagoe et al., 1993; Perdew et al., 1993; Morimoto et al., 1994; Quraishi & Brown, 1995).

In nonneuronal systems, HSP90 proteins help regulate intracellular protein activity, including protein transport (Pratt, 1993; Bohen & Yamamoto, 1994; Smith et al., 1995; Johnson et al., 1996). HSP90 proteins associate with specific tyrosine and serine kinases (Xu & Lindquist, 1993; Mimnaugh et al., 1995), tubulin, actin, and calmodulin (Koyasu et al., 1986; Nishida et al., 1986; Sanchez et al., 1988), and play an important role in steroid receptor activation (for review see Pratt, 1993). Loss of HSP90 activity inhibits the cellular response to both the nerve growth factor (NGF)-mediated signaling pathway, as well as *v-src* kinase activity (Xu & Lindquist, 1993; Jaiswal et al., 1996), suggesting a role for HSP90 in the regulation of these pathways. HSP90 protein associations can also alter intracellular protein activation and function (Picard et al., 1990; Xu & Lindquist, 1993; Nathan & Lindquist, 1995).

HSP90 mRNAs and protein are constitutively expressed at high levels in neural tissue, including retina and brain (Izumoto & Herbert, 1993; Gass et al., 1994; Tanaka et al., 1995). This suggests that HSP90 proteins may play a selective role in neural function. We previously found that HSP90 mRNA is expressed at higher levels in total RNA from the central region of the primate retina (the fovea), as compared to the retinal periphery (Bernstein et al., 1995, 1996). HSP90 mRNAs are selectively expressed in rodent retinal ganglion cells (RGCs) (Tanaka et al., 1995). Since RGCs are concentrated in the fovea region of primate retina (Shapely & Perry, 1986; Ogden, 1994), it is likely that HSP90 plays an RGC-specific or intensive function.

The RGC axon comprises the major portion of the optic nerve (Dacheux & Raviola, 1994; Varma & Minckler, 1996). RGC

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axoplasm may comprise up to 94% of RGC cytoplasmic volume (Varma & Minckler, 1996). Because HSP90 proteins play a role in nonneuronal intracellular protein transport, we hypothesized that HSP90 is utilized in intra-axonal transport.

RGC neurons also synthesize full-length tropomyosin related kinase B (fl-trkB) and trkC (Takahashi et al., 1993; Hallbook et al., 1995). Fl-trkB is axonally transported through the optic nerve to the presynaptic membrane in the central nervous system (CNS), where it binds to CNS-secreted, brain-derived growth factor (BDNF) (Barde et al., 1987; Herzog et al., 1994; Meyer-Franke et al., 1995). TrkB-BDNF binding in the CNS results in trkB dimerization, autocatalysis, and autophosphorylation of specific tyrosine residues in the trkB molecule (Jing et al., 1992). Fl-trkB autocatalysis is the initial step in a signal chain required for RGC neuron survival (Jing et al., 1992). TrkC binds neurotrophins-3 (Lewin et al., 1996). The BDNF-activated trkB complex is believed to be retrograde axon transported to the RGC soma (Dechant et al., 1994; Kaplan & Stephens, 1994; Lewin & Barde, 1996; Johanson et al., 1996; Johnson et al., 1996). Trk activation also initiates presynaptic MAPK kinase (MEK) and mitosis associated phosphokinase (MAP) and is associated with MEK/MAP kinase phosphorylation (Cowley et al., 1994; Hill & Treisman, 1995). Activated MAP kinases are retrograde axon transported to the cell nucleus, and can substitute for the trk-neurotrophin primary event, suggesting that they participate as part of the secondary neurotrophin signal pathway (Cowley et al., 1994; Hill & Treisman, 1995). Although it has been demonstrated that HSP90 is associated with MAP kinase function (Jaiswal et al., 1996), it is unknown whether HSP90 is directly associated with the active or inactive form of MAP kinase. An intra-axonal association of HSP90 with activated MAP/MEK would suggest that HSP90 plays a role in both anterograde and retrograde axonal protein transport, directly implying a neuron-specific role for HSP90 in synaptic function and retinal ganglion cell survival.

Methods

Materials

Rhesus retina tissue was obtained from freshly euthanized (24–36 month old) monkeys previously utilized in vaccine studies (a kind gift of Dr. J. Cogan, Center for Biologics evaluation and research, U.S. Food and Drug Administration). Before dissection, eyes were examined grossly for signs of ocular disease. Bovine retinae and optic nerves were obtained from a local abbatoir, freshly dissected from the globe and snap-frozen on dry ice. Primate retina used for *in situ* or immunohistological analysis was briefly fixed in 4% paraformaldehyde-Dulbeccos phosphate buffer (D-PBS) and either frozen in OCT freezing medium or embedded in paraffin. Tissues were stored at −70°C prior to use.

Immunochemicals

Polyclonal isoform-specific antibodies to HSP90β and HSP90α were purchased from ABR (Golden, CO). Rabbit polyclonal antibodies to trkB, trkB (tk-), trkC, and mouse anti-phosphotyrosine monoclonal antibody (py-99) were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). Mouse monoclonal antibody to phosphorylated MAP kinase (pp42/44) and kinase assay kit were purchased from New England Biolabs (Boston, MA). Polyclonal chicken IgY pan-trk antibody, and antibodies to mammalian BDNF and NT-3, were obtained from Promega (Madison, WI).

In situ hybridization

In situ hybridization was performed using sections of rhesus and human retinal tissue prepared as described for mouse (Pierce et al., 1995). An antisense 385 nt digoxin-labeled cRNA fragment was transcribed from a PCR-generated HSP90β cDNA template fragment (Genbank accession number X15183), engineered with flanking T_3 (sense) and T_7 (antisense) RNA polymerase promotor sequences. The HSP90 sense strand (control) coding sequence was 5′ GAC GTT CGC CTT TCA GGC AGA. The 3′ Antisense strand coding sequence was 5′ TCT CAG CAA CCA AAT AAG CA 3′.

Western analysis

Rhesus and bovine tissue homogenates were prepared and protein concentrations determined using the Bradford reaction. For western analysis, equivalent amounts of protein homogenate were loaded per well. Polyacrylamide gel electrophoresis (PAGE) was performed on protein homogenates using the Phast system (Pharmacia Biotech, Piscataway, NJ). Following PAGE, proteins were either transferred to nitrocellulose or polyvinyldifluoride (PVDF) membranes, using the manufacturers recommended protocols. Membranes were blocked in I-block (Tropix; Waltham, MA), and reacted with the appropriate antibody. Blots were developed using a primary antibody species-specific alkaline phosphatase-linked secondary antibody, and the CSPD chemiluminescence system (Tropix; Waltham, MA). Western analysis using a mouse monoclonal antibody to phosphorylated MAP kinase was performed in a similar fashion, except that 5% nonfat milk was substituted in place of I-block.

Immunohistology of rhesus retina and optic nerve

Immunohistological analysis was performed on fixed frozen sections of rhesus retina and optic nerve, using HSP90α- and HSP90β-specific antibodies (ABR; Golden, CO). Treated sections were incubated with either alkaline-phosphatase secondary antibody (retina; Vector Laboratories, Burlingame, CA), or fast red (optic nerve) and counterstained with hematoxylin (retina).

Co-immunoprecipitation of HSP90 proteins

Freshly obtained bovine optic nerve was homogenized on ice in RIPA buffer (phosphate buffered saline pH 7.4; 15 mM $MgCl_2$; 1 mM DTT; 0.2% SDS; 0.4% NP-40; 1 mM each of aprotinin, leupeptin, pepstatin, 500 micromolar AEBSF, and 300 mM sodium orthovanadate). Homogenates were precleared by adding 1 μg/ml normal rabbit IgG (Jackson Immunoresearch; Hershey, PA), and incubating on ice with protein-G sepharose (Pharmacia Biotech). HSP90α- or HSP90β specific antibodies were added to the precleared homogenates, and incubated an additional 3 h. Immunoprecipitation was performed using protein-G sepharose beads (Pharmacia). Immunoglobulin-bound protein complexes were extensively washed with RIPA, and either frozen at −70°C, or used immediately for kinase assays.

Chemiluminescent MAP kinase assay

Activated MAP kinase activity was assayed using a chemiluminescent MAP kinase assay (NEB; Waltham, MA). Co-immunoprecipitates were incubated with elf-1 fusion protein substrate, along with 15 mM cold ATP, in kinase buffer (10 mM $MgCl_2$;

2 mM DTT; 50 mM Tris-HCl (pH 6.8), 10 mM orthovanadate, 1 mM each aprotinin, leupeptin, and pepstatin). For the calcium release experiments, immunoprecipitates were incubated at 37°C in assay buffer containing 0–30 mM of calcium chloride. The immunoprecipitates and supernatant were then assayed using the chemiluminescent kinase assay (NEB). Reaction products were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with the appropriate rabbit polyclonal antibody. Blots were developed using a biotinylated goat-antirabbit antibody-horseradish peroxidase (HRP) linked chemiluminescent system (NEB).

Results

HSP90 expression in RGCs

HSP90 expression in rhesus monkey retinal tissue was evaluated by *in situ* hybridization, using a digoxin-labeled HSP90β cRNA transcript as probe. The strongest HSP90β signal is found over the RGC layer (Fig. 1a; GCL). A weaker but relatively strong signal is also generated in the photoreceptor inner segment region (Fig. 1a; IS) and the outer nuclear layer (ONL), which consists mainly of photoreceptor neuron nuclei (rods and cones). No signal is seen when retina is reacted with the control (sense) probe (Fig. 1b).

HSP90α and HSP90β protein in retina and optic nerve

Expression of HSP90 isoforms (90α and 90β) in the rhesus retina and optic nerve were performed using western analysis and immunolocalization (Fig. 2). As shown in Fig. 2a, HSP90α and HSP90β signal intensities are higher, per microgram of total tissue, in retina than in the optic nerve (Fig. 2a; compare R and ON).

Immunohistologic analysis of rhesus retina using antibodies to both 90α and 90β shows that HSP90β immunoreactivity is expressed at high levels in retinal layers associated with RGC neurons, particularly the nerve fiber layer (NFL) (Fig. 2b). The NFL comprises the intraretinal RGC axon component. HSP90α is expressed throughout the retina, including the outer nuclear (photoreceptor cell nuclei) layer [Fig. 2b (90α); ONL]. Immunoreactivity is not seen in the control section (Fig. 2b; control). HSP90α and HSP90β immunostaining of rhesus optic nerve is seen in Fig. 2c (90β and 90α). Strong immunoreactivity is seen in axon fascicles for both isoforms (Fig. 2c; 90α and 90β; Ax). The optic nerve adventitia is also strongly positive for both HSP90 isoforms (Fig. 2c; Ps.). These results suggest that HSP90β and HSP90α proteins are associated with the intraretinal and extraretinal axon compartment of retinal ganglion cells, as well as the supporting adventitial cells.

Optic nerve HSP90 exists as 90α/90β heterodimers

Co-immunoprecipitation experiments utilizing antibodies to either HSP90 isoform (90α or 90β), generate a strong signal for both isoforms, when the appropriate antibody is used for detection (Fig. 3). A strong HSP90α signal is detected from the HSP90β co-immunoprecipitate (Fig. 3a; compare 90α and 90β lane), suggesting that, similar to other cellular systems (Perdew et al., 1993), 90β and 90α isoforms exist in the optic nerve as α/β heteroduplexes.

HSP90 associations with neurotrophin signaling proteins

Because optic nerve full-length trkB (fl-trkB) originates in RGCs (Allendoerfer et al., 1994; Garner et al., 1996), isolated optic nerve fl-trkB directly reflects RGC-axon transport. HSP90 isoform and trk-specific co-immunoprecipitates were prepared from bovine optic nerve homogenates and screened by western analysis, using antibodies to fl-trkB (Fig. 4). Fl-trkB is detectable by western analysis in the rat brain control (Fig. 4a; RBC), and at levels in unconcentrated bovine optic nerve homogenate (Fig. 4a; RIPA) that are only detectable using western signal generation exposure times far longer (2 h) than that used for HSP90 co-immunoprecipitates (1 h). In comparison, western analysis of HSP90 isoform co-immunoprecipitates generate a relatively strong signal for the full-length, 145-kDa trkB species (compare the trkB signal from HSP90α and HSP90β co-immunoprecipitates, Fig. 4A, with the trkB signal generated from RIPA/optic nerve homogenate, Fig. 4A). Minimal HSP90 is associated with adventitial trkB (tk-) forms (95-kDa forms; data not shown). TrkB signal is absent when co-immunoprecipitates are prepared at room temperature, in the absence of 15 mM $MgCl_2$, or after incubation of the co-immunoprecipitates at 50°C (data not shown), suggesting that HSP90-TrkB association is not an artifact of the immunoprecipitation procedure, but rather occurs intra-axonally, *in vivo*.

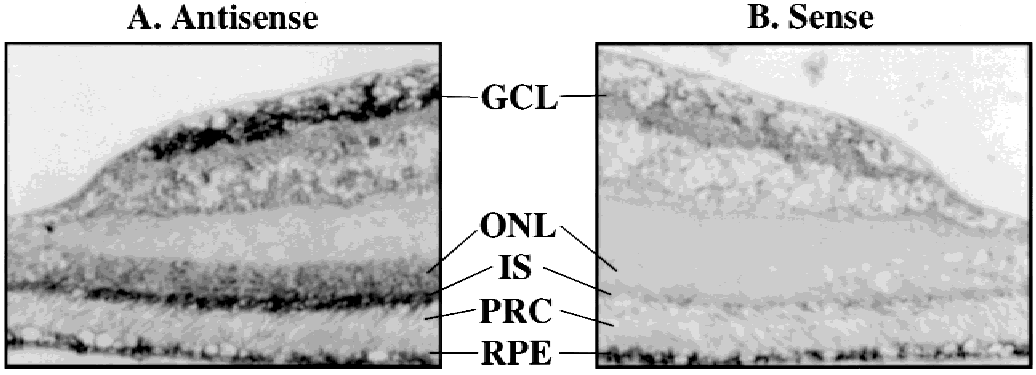

Fig. 1. *In situ* analysis of HSP90β mRNA in the rhesus retina. A digoxigenin-labeled cRNA probe was transcribed using either T_3 or T_7 RNA polymerase as described in Methods. The RNA probes were hybridized to 8-micron-thick sections of rhesus retinal fovea tissue. A: antisense (experimental) probe. B: sense (control) probe. GCL: ganglion cell layer; ONL: outer nuclear (photoreceptor nuclei) layer; IS: inner segment of the photoreceptor; PRC: photoreceptor layer; and RPE: retinal pigment epithelial layer. The brown coloration of the RPE layer is due to melanin. Magnification: 320×.

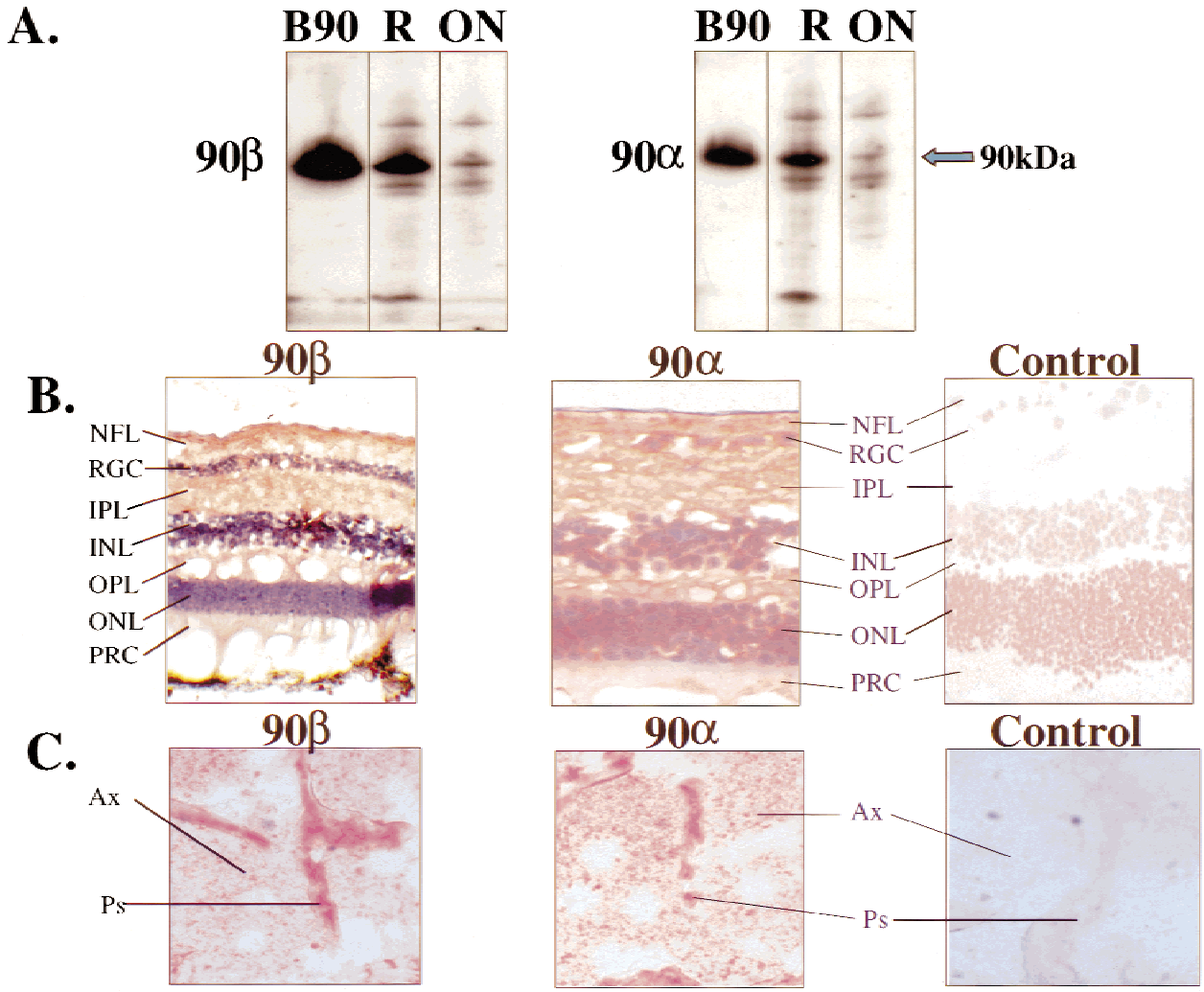

Fig. 2. HSP90α and 90β expression in bovine retina and optic nerve. A: Western analysis of bovine retina (R) and optic nerve (ON). Equal amounts of tissue homogenates (by Bradford reaction) were electrophoresed on 7.5% polyacrylamide-SDS-tricine gels using the PHAST system. Control bovine HSP90 protein (B90) was co-electrophoresed with the homogenates (ABR). Duplicate gels were transferred to nitrocellulose membranes (S & S), reacted with the appropriate antibody and developed as described in Methods. Exposure time: 10 min. 90α: Membrane reacted with rabbit anti-HSP90α. 90β: Membrane reacted with rabbit anti-HSP90β. B: Immunohistolocalization of HSP90α and HSP90β isoforms in the rhesus retina. Frozen sections were reacted with antibodies to either HSP90α (90α) or HSP90β (90β). Tissue was reacted with biotinylated secondary antibody, treated with peroxidase/AEC substrate (ABC; Vector Laboratories; CA), and counterstained with Hematoxylin. NFL: nerve fiber layer; RGC: retinal ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; and PRC: photoreceptor layer. Magnification: 90β at 160× and 90β at 400×. Control: 160×. C: Immunohistolocalization of HSP90α and HSP90β isoforms in the rhesus optic nerve. Frozen sections of optic nerve were reacted with antibodies to either HSP90α (90α) or HSP90β (90β). Tissue was reacted with biotinylated secondary antibody, and treated with alkaline phosphatase-avidin and fast red (Extra-avidin; Sigma). Ax: axon bundle. Ps: adventitial layer (Pial sheath). Magnification: 320×

RGC-synthesized trkC has been shown to be expressed in the rat optic nerve, retina, and tectum (Allendoerfer et al., 1994; Rickman & Brecha, 1995). Fl-trkC is detectable in western analysis of bovine optic nerve homogenates (Fig. 4c; RIPA) and trkC-specific bovine optic nerve immunoprecipitates (Fig. 4b; trkC). When trkC-specific antibody is used for co-immunoprecipitation (Fig. 4c), no western signal for either HSP90α (Fig. 4c; trkC$^{\alpha}$) or HSP90β (Fig. 4c; trkC$^{\beta}$) is detectable. These results suggest that, in the bovine optic nerve, HSP90α and HSP90β may associate specifically *in vivo* with fl-trkB.

HSP90 associates with the nonactivated form of trkB

Activated (phosphotyrosine positive) trkB bound-BDNF complex is believed to be transported by retrograde axonal transport by retinal ganglion cells *in vivo* (Kaplan & Stephens, 1993; Johnson et al., 1996). Fl-trkB apparently synthesized by RGCs is detectable by HSP90 co-immunoprecipitates (see Fig. 4a; 90α and 90β). If activated fl-trkB associates with HSP90 during retrograde transport, HSP90 co-immunoprecipitates should generate a phosphotyrosine-positive signal at 145 kDa (fl-trkB).

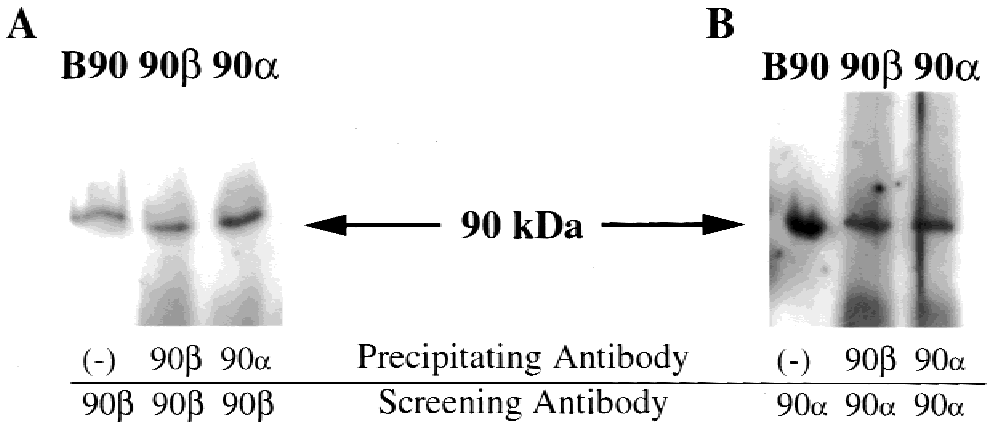

Fig. 3. Co-immunoprecipitation of HSP90 isoforms in the bovine optic nerve. The lower table indicates the screening strategy. Tissue was homogenized in RIPA buffer and immunoprecipitated with either anti-HSP90α or HSP90β as described in Methods. Immunoprecipitates were electrophoresed on 7.5% SDS-PAGE and transferred to nitrocellulose membranes (S & S). 3A: membrane screened with antibody to HSP90β. 3B: membrane screened with antibody to HSP90α. Membranes were developed using a CSPD-biotin-linked chemiluminescence system (Tropix). Lane identification: 90β; homogenate immunoprecipitated with HSP90β antibody. 90α; homogenate immunoprecipitated with HSP90α antibody. B90: purified bovine brain HSP90 (control) protein. Reaction time: 10 min. The molecular weight is indicated by an arrow.

Bovine optic nerve HSP90 co-immunoprecipitates, optic nerve homogenate, and an EGF-stimulated rat brain control (upstate technologies) were screened with py-99 antiphosphotyrosine antibody (Fig. 5c). To independently determine whether a portion of the HSP90 bound fl-trkB is active, we also screened the HSP90 co-immunoprecipitates for BDNF using a chicken anti-BDNF-specific antibody (Promega, Madison, WI) (Fig. 5b). Both bovine optic nerve homogenate and EGF-stimulated controls generate a detectable phosphotyrosine signal (Fig. 5c; RIPA and Fig. 5b; control). No signal is detectable in the HSP90 co-immunoprecipitates using phosphotyrosine antibody (py-99) (Fig. 5a). The anti-BDNF antibody generates a signal when reacted against bovine retina (Fig. 5b; Retina) and bovine optic nerve homogenate (Fig. 5b; RIPA). No BDNF signal is detectable in the trkB-positive, HSP90 α/β-bovine optic nerve immunoprecipitates (Fig. 5b; 90α and 90β), suggesting that, while BDNF is present in retina and optic nerve, it is not associated with fl-trkB bound to HSP90. These results suggest that only unactivated fl-trkB is associated with HSP90 in the optic nerve and retina.

Fl-trkC is demonstrable in the retina and optic nerve homogenates and can be directly immunoprecipitated by anti-trkC antibody (see Fig. 4B; trkC). TrkC binds neurotrophins-3 (NT-3). HSP90 co-immunoprecipitates, retina homogenate, and optic nerve homogenate were reacted with an antibody specific for neurotrophins-3 antibody (Fig. 5a). A signal for NT-3 is generated by the bovine optic nerve (Fig. 5a; RIPA) and retina homogenates (Fig. 5a; Retina). In contrast, no NT-3 signal is seen in HSP90α or HSP90β immunoprecipitates (Fig. 5a; 90α and 90β).

HSP90-MAP kinase associations in bovine optic nerve

TrkB-neurotrophin binding results in MAP kinase activation (Hill & Treisman, 1995). Activated MAP kinases are retrograde axon transported to the neuron nucleus (Chen et al., 1992). To determine

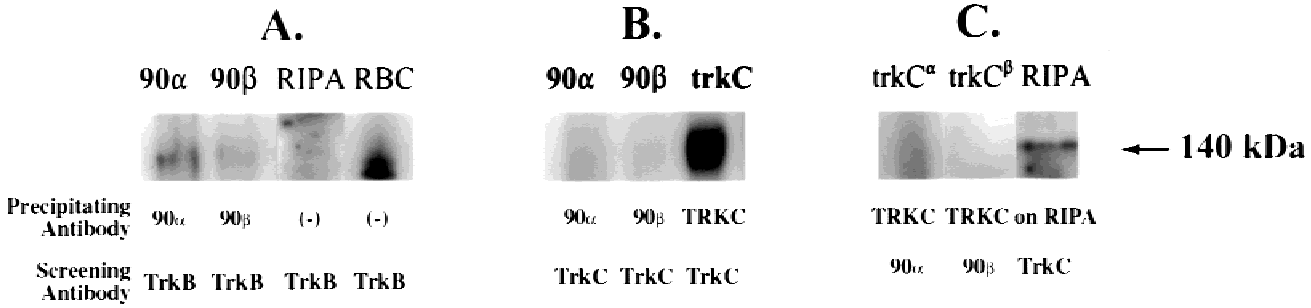

Fig. 4. Trk analysis in HSP90 and trkC immunoprecipitates. Immunoprecipitates were prepared from bovine optic homogenates using antibodies to (A) HSP90α and HSP90β, and screened with antibody to trkB. RIPA: bovine optic nerve homogenate. RBC: rat brain homogenate (control). (B) trk (sc-112), and screened with antibody to either HSP90α (90α), HSP90β, or trkC. (C) Immunoprecipitated with trkC, and screened with antiHSP90α (TrkC$^{\alpha}$). Immunoprecipitated with trkC, and screened with antiHSP90β (TrkC$^{\beta}$): RIPA: bovine optic nerve homogenate, screened with trkC. Immunoprecipitates were prepared from bovine optic nerve as described in Methods. Exposure times: 2 h (Fig. 4A; RIPA homogenate). One hour all other lanes.

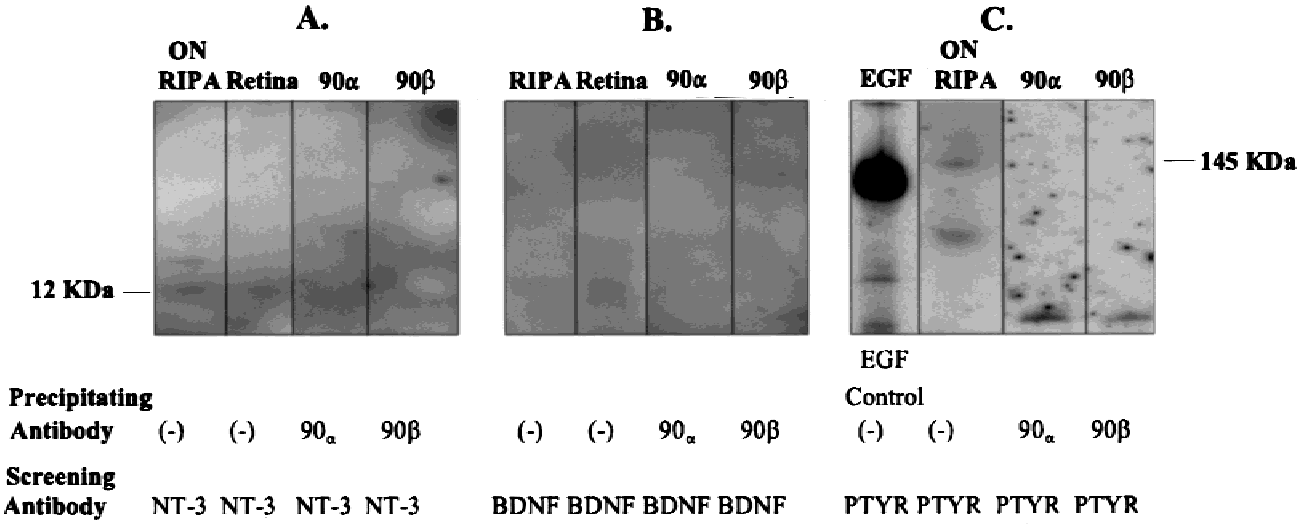

Fig. 5. Bovine optic nerve immunoprecipitates screened using antibodies to (A) BDNF, (B) phosphotyrosine, and (C) NT-3. Samples analyzed for neurotrophins were transferred to PVDF membranes; precipitates screened for phosphotyrosine were transferred to nitrocellulose (S & S). Membranes were blocked with I-block, reacted with the appropriate antibody, and developed using the CSPD chemiluminescent system (Tropix). 90α: 90α immunoprecipitate. 90β: 90β immunoprecipitate. BON: Bovine optic nerve protein homogenate. Retina: bovine retina homogenate. EGF: positive phosphotyrosine control (EGF stimulated rat fibroblast homogenate). Exposure times: A: 3 min; B: 1 h; and C: 1 h. Molecular weights are indicated.

whether HSP90 is utilized in both anterograde and retrograde axonal transport, HSP90 co-immunoprecipitates were screened for the presence of phosphorylated p42 MAP kinase, using a phospho-MAP antibody (Fig. 6). A minimal signal for phosphorylated MAP kinase is generated by the bovine optic nerve homogenates (Fig. 6a; RIPA). In contrast, a strong signal for phosphorylated MAP kinase is detectable in both HSP90α and HSP90β bovine optic nerve immunoprecipitates (Fig. 6a; 90α and 90β), suggesting that RGC axonal HSP90 associates with activated (retrograde transported) MAP kinase.

To identify whether MAP kinase is inactivated during its association with HSP90, an *in vitro* phosphorylation assay was performed (Fig. 6b). HSP90α and HSP90β optic nerve co-immunoprecipitates directly phosphorylate an appropriate substrate (Fig. 6b; HSP90α and HSP90β), suggesting that HSP90-bound phosphorylated MAP kinase is active; HSP90 association does not prevent MAP function. Incubating HSP90 co-immunoprecipitates with kinase assay buffer containing calcium (0–30 mM) does not release MAP kinase from the HSP90 co-immunoprecipitate (data not shown). Release does occur after heating (48°C/10 min; data not shown).

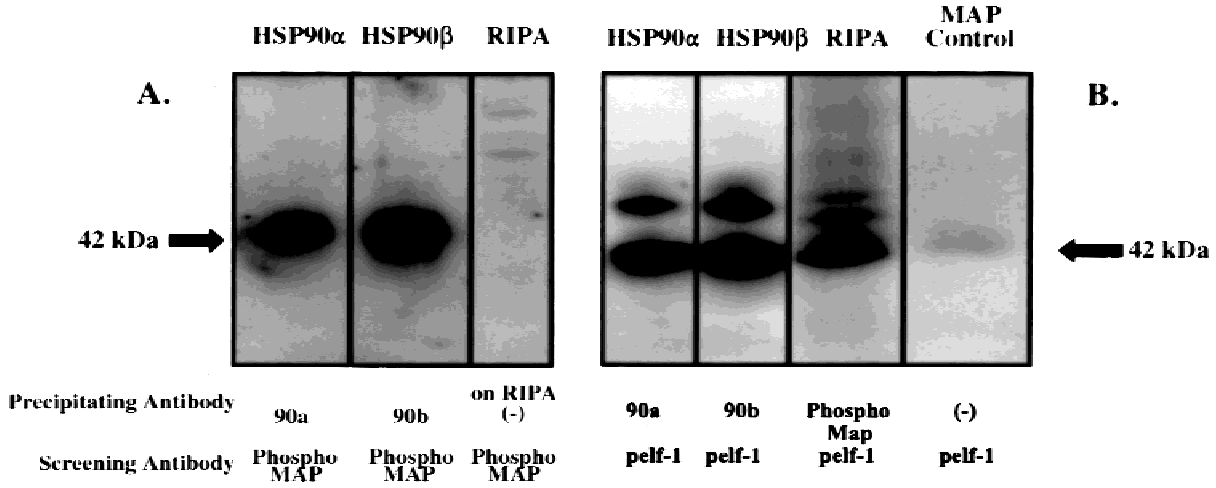

Fig. 6. HSP90 association with phosphorylated, active optic nerve MAP kinase. A: Western analysis of HSP90 optic nerve immunoprecipitates with antibody to phosphorylated MAP kinase. HSP90α (HSP90α) and HSP90β (HSP90β) immunoprecipitates were electrophoresed, transferred to nitrocellulose, and reacted with antibody to phosphorylated (p42) MAP kinase (NEB). Blots were developed using the CSPD system (Tropix). Exposure time: 10 min. B: MAP kinase assay of HSP90 immunoprecipitates. HSP90α (HSP90α) and HSP90β (HSP90β) immunoprecipitates were functionally evaluated using a chemiluminescent kinase assay (NEB). Optic nerve-HSP90 immunoprecipitates were incubated with cold ATP and elf-1 protein as described in Methods. The reaction products were electrophoresed and analyzed using an elf-1 phospho-specific antibody (NEB). Exposure time: 10 min.

Discussion

This is the first study to identify HSP90 as a participant in RGC axonal transport. HSP90 proteins were previously believed to be absent in the axonal compartment (Gass et al., 1994). This disparity in previous reports is likely due to an artifactual reduction of HSP90 immune signal in formalin- or paraformaldehyde-fixed tissues.

HSP90 mRNA is found at highest intraretinal levels in RGCs (Fig. 1a). This transcriptional activity results in elevated levels of translated HSP90 proteins, both in the RGC-associated retinal layers (Figs. 2a and 2b; 90α and 90β), and in the optic nerve-associated RGC axonal compartment (Figs. 2c; 90α and 90β). While optic nerve HSP90 levels are apparently lower per gram wet weight, in optic nerve than in retina, it must be recognized that up to 95% of the RGC cytoplasm is present in the axonal compartment (Ogden, 1994). The vast majority of RGC-expressed HSP90 is thus likely to be found in the optic nerve/optic tract compartment. Our findings thus functionally correlate with previous reports of high-level mRNA expression (differential expression) in RGCs and RGC-enriched primate retinal regions (Ogden, 1994; Bernstein et al., 1995, 1996).

Immunoprecipitation with either 90α or 90β results in the isolation of the alternate HSP90 isoform (compare Figs. 3a and 3b), implying that much RGC-expressed HSP90 is present as 90α/90β heterodimers. Retinal HSP90 isoform expression is therefore consistent with that seen in nonneural systems (Perdew et al., 1993; Gruppi & Wohlgemuth, 1993).

Fl-trkB and trkC are synthesized exclusively by RGCs (Allendoerfer et al., 1994; Hallbook et al., 1995). Following trk activation at the axon terminal synapse, the trk-neurotrophin complex is believed to be retrograde transported (Meyer-Franke et al., 1995; Hayashi, 1996; Herzog & von Bartheld, 1998). Fl-trkB levels in unconcentrated optic nerve homogenate are very low (Fig. 4a; RIPA). Fl-trkB is clearly detectable in HSP90 co-immunoprecipitates, suggesting that it associates with (and can be concentrated by) copurification with HSP90. Since intra-optic nerve fl-trkB can be assumed to be intra-axonal, the association of fl-trkB with HSP90 suggests that HSP90 may be utilized for axonal transport.

BDNF is not detectable in the trkB-HSP90 co-immunoprecipitates (Fig. 5b). Similarly, HSP90-bound trkB co-immunoprecipitates are unphosphorylated (Fig. 5c; 90α and 90β). Thus, it can be hypothesized that HSP90 proteins associate mainly with inactive (unphosphorylated/non-BDNF associated) fl-trkB. This suggests the possibility that the trkB-HSP90 association may occur primarily during anterograde axonal transport. Activated trkB may be retrograde transported in the axon without HSP90; activated fl-trkB is detectable in bovine optic nerve homogenates and co-immunoprecipitates utilizing antiphosphotyrosine antibody (data not shown). Direct evidence of HSP90's association with fl-trkB in anterograde axonal transport, however, is lacking.

TrkC is present in bovine optic nerve homogenate (Fig. 4c; RIPA), and can be directly precipitated with trkC antibody (Fig. 4b; trkC). The lack of trkC-HSP90 association (Fig. 4b; 90α and 90β) is therefore surprising, since RGC-synthesized fl-trkC is apparently transported to the CNS axon terminal synapse (Allendoerfer et al., 1994; Hallbook et al., 1995). A fl-trkB signal can be detected in the same HSP90 immunoprecipitates used for trkC (Fig. 4a; 90α and 90β). HSP90 may be selectively utilized for trk species anterograde axonal transport, similar to HSP90 associations in nonneuronal systems that distinguish between related but distinct hormone receptor classes (Dalman et al., 1991).

High levels of phosphorylated, optic nerve MAP kinase are associated with HSP90α and HSP90β (Fig. 6a). MAP kinase activation can substitute for the neurotrophin neuronal requirement (Cowley et al., 1994; Hill & Treisman, 1995), and activated MAP kinase is retrograde transported to the cell nucleus (Chen et al., 1992), suggesting that MAP kinase activation is a secondary response to trk phosphorylation. The HSP90-associated, phosphorylated MAP kinase is functionally active, and can phosphorylate protein substrates *in vitro* (Fig. 6b), suggesting that HSP90 association does not prevent MAP kinase function. Thus, our results are consistent with a model in which MAP kinase (activated at the axon terminal synapse) binds to HSP90 during retrograde axon transport. The *in vivo* mechanism regulating HSP90-protein association and dissociation is unknown. Varying the calcium ion concentration from 5–30 mM does not release either trkB or MAP kinase from HSP90 immunoprecipitates *in vitro* (data not shown). Thus, intracellular calcium concentration does not, by itself, control HSP90-protein dissociation.

The intra-axonal association of HSP90 with trkB and activated MAP kinase suggests that HSP90 is a component of axonal neurotrophin receptor and signal transport pathways. Previous reports have shown that receptor tyrosine-kinase signaling (Cutforth & Rubin, 1994) and MAP kinase-protein interactions require HSP90 (Jaiswal et al., 1996). These pathways are postulated to be restricted in ischemic optic neuropathies such as glaucoma, resulting in RGC cell death (Kaplan & Stephens, 1994; Unoki et al., 1994; Meyer-Franke et al., 1995; Quigley et al., 1995; Johnson et al., 1996). HSP90 mRNA levels decrease in glaucomatous retinae (data not shown). Disease-induced reduction in HSP90 expression may contribute to a relative deficiency of neurotrophin-based signaling to the RGC nucleus, causing RGC death. Constitutive high-level expression of HSP90 isoforms, and selective induction in stress-related events, may form one of the bases for resistance to genetic and acquired ganglion cell disease.

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