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Sensory Denervation Modulates eIF-2 Alpha Kinase Expression in the Rabbit Lacrimal Gland

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Abstract

Purpose—To investigate the hypothesis that sensory denervation of the rabbit lacrimal gland results in dysregulation of protein synthesis. We used differential display of mRNA to identify genes associated with protein synthesis and secretion that may be altered in this situation.

Methods—New Zealand white rabbits underwent unilateral sensory denervation by the ablation of the trigeminal ganglion. After 7 days, the denervated and contralateral control lacrimal glands were removed. The effects of denervation on gene expression were carried out using differential mRNA display. Northern and Western blot analyses were used to verify differential gene expression.

Results—Differential mRNA display identified the gene heme-regulated inhibitor eukaryotic initiation factor-2 alpha kinase (HRI eIF-2 α kinase) in the lacrimal gland, the expression of which was reduced in the denervated lacrimal gland. The sequenced fragment from differential display showed 94% identity to rabbit HRI eIF-2 α kinase. The decreased expression of HRI eIF-2 α kinase was confirmed by Northern and Western blots, and measurement of HRI eIF-2 α kinase phosphorylation activity in the lacrimal gland after ablation of sensory neurons showed that it was significantly decreased compared with that of normal and control lacrimal glands.

Conclusions—The results suggest that loss of sensory innervation has a role in the lacrimal gland, contributing to the expression of HRI eIF-2 α kinase, a pivotal negative regulator of protein synthesis. A reduction in control of protein synthesis may lead to the translation of repressed messages associated with cell stress responses.

Keywords

denervation; differential mRNA display; eIF-2 α kinase; lacrimal gland

INTRODUCTION

The lacrimal gland (LG) is the major secretory source for proteins and electrolytes found in normal tears. The functions of this dilute protein solution are to optimize the optics of the cornea, to lubricate, and to protect the eye from pathogens.¹ Secretion of lacrimal fluid and proteins is under neural control, with the participation of neural pathways activated from the

cornea, as well as central trigeminal pathways coupled to efferent parasympathetic fibers.^{2,3} It has been suggested that both normal and reflexive tear flow require the participation of neural components.^{1,3,4}

It is known that selective ablation of trigeminal ganglion (TG) neurons subserving the cornea results in an alteration of corneal epithelial cell function, which may be due in part to changes in tear quality.⁵ In rabbits with a trigeminal ganglion lesion, pharmacologic stimulation of the lacrimal gland *in vitro* by the muscarinic cholinergic agonist carbachol or the β -adrenergic agonist isoproterenol was found to increase protein release, and this phenomenon was shown to be membrane receptor-dependent.⁶ These findings correlate with electron microscopic observation of the accumulation of secretory granules in the denervated lacrimal gland,⁷ suggesting that although granules are synthesized *in vivo*, the mechanism for release is compromised. Therefore, accumulation of secretory granules may be the result of decreased activation of the muscarinic, parasympathetic neural pathway.⁷

Dysfunction of the main secretory pathway can also affect expression levels within the endoplasmic reticulum associated with post-translational processing of secretory proteins.⁸ *In vitro* analysis of protein synthesis using purified acinar cells from sensory denervated LG showed that translation reduced regulatory inhibition compared with the normally innervated, contralateral control LG.⁹ Furthermore, matrix-assisted laser desorption/ionization–mass spectrometry (MALDI-MS) of rabbit tears found differences in the tear protein profiles of denervated and contralateral normal LG.⁹ These results suggest that lacrimal secretory protein synthesis may be affected by sensory denervation, possibly from decreased expression or activity of factor(s) that regulate protein synthesis.

Because we observed an accumulation of secretory vesicles and increased *in vitro* release of secretory protein after sensory denervation, we hypothesized that the mechanism regulating secretory protein synthesis may be compromised in the LG. In the current study, we used differential mRNA display to identify genes whose expression was changed in the rabbit LG after short-term sensory denervation.

MATERIALS AND METHODS

Animals

Male New Zealand white rabbits (2–3 kg) were purchased from a local supplier (LSUHSC, New Orleans, LA, USA). Animals were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the experimental protocol used in this study was approved by the Institutional Animal Care and Use Committee (IACUC) of the Louisiana State University Health Sciences Center.

Sensory Denervation

Controlled, unilateral sensory denervation of the rabbit lacrimal gland was carried out as previously described by ablation of the TG neurons subserving the sensory nerves to the lacrimal gland and cornea.⁶ After the procedure, the eyes were taped shut until the animals recovered from the anesthesia. All animals received food and water *ad libitum* after the procedure. The animals were examined on a daily basis. To ensure that the denervation procedure was complete, the absence of the blink response was confirmed by lightly touching the cornea with a cotton wisp. Seven days after surgery, the animals were sacrificed, and the lacrimal glands were removed from the contralateral intact control and denervated sides.

Sympathetic denervation was carried out as previously described.¹⁰ In brief, unilateral removal of the superior cervical ganglion was carried out after the identification of pre- and postganglionic processes. Animals were sacrificed after 7 days.

Differential mRNA Display

Total RNA was obtained by guanidium thiocyanate/cesium chloride ultracentrifugation as described previously.¹¹ Primers were designed (Table 1), and detailed protocols for the differential mRNA display analysis of DNase-treated total RNA were performed as described.^{12,13} The mRNA profiles from contralateral and denervated lacrimal glands were compared from two separate denervation studies. For DNA sequencing, the reamplified product was separated on a 1% agarose gel, excised, and isolated using a microconcentrator (Microcon; Amicon Inc., Beverly, MA, USA). The amplicon was quantitated by spectrophotometry, and cycle sequencing was carried out using [³⁵S]dATP (1500 Ci/mmol; Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's directions (Episcience, Madison, WI, USA) with an additional annealing step set at 42°C. The sequencing primer was the same as the primers used in differential mRNA display. The DNA sequence was submitted for homology analysis using Blastn (National Center for Biotechnology Information, Bethesda, MD, USA).¹⁴

Northern Blot Analysis

A 20-μg RNA sample was separated on 1.2% agarose/formaldehyde denaturing gels and transferred to Zeta Probe membranes (Biorad, Hercules, CA, USA). Prehybridization was done at 42°C with the addition of heat-denatured salmon sperm DNA (100 μg/ml; Sigma, St. Louis, MO, USA) for at least 2 hr. The gel-purified eIF-2α kinase (100 pg) was labeled with [α-³²P]-dCTP (Amersham) by PCR using the same parameters as were used for differential mRNA display and purified using a G25 Sephadex column (Biorad). The control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH; clone PHcGAP), was purchased from American Type Culture Collection (Manassas, VA, USA) and radiolabeled in the same manner except that GAPDH-specific primers¹¹ were used in a separate PCR reaction (90°C, 50°C, 72°C, 1 min each cycle for 30 cycles, then 72°C for 5 min). DNA probes (5 × 10⁶ cpm/ml) were used for the overnight hybridization at 42°C. Blots were washed in decreasing concentrations of SSC (2×, 0.5×, 0.1× SSC/0.1% SDS) for 15 min each and exposed overnight at -70°C (Kodak X-AR; Kodak, Rochester, NY, USA).

Western Blot Analysis

The procedures for Western blot analysis were carried out as previously described.¹⁵ In brief, frozen lacrimal glands were homogenized in hot 10 mM Tris-Cl (pH 7.4) with 1% SDS, and total protein lysate was quantified using a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL, USA). Equal amounts (50 μg) of protein were separated on 7.5% polyacrylamide gels and transferred overnight at 4°C to nitrocellulose membranes (Hybond-ECL; Amersham). Nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI, USA) was used as a positive control for HRI. The membrane was blocked in a 5% solution of nonfat powdered milk, then incubated with anti-p74 HRI (1:500) followed by anti-mouse horseradish peroxidase secondary antibody (1:2000, Amersham) for 1 hr each. The anti-74 HRI monoclonal antibodies were made at the LSU Health Sciences Center Core Laboratories using a synthetic p74 sequence (SDMYSVGVLLELFQPFGE) corresponding to the kinase activation domain (IX) of rabbit HRI eIF-2α kinase.¹⁶ The specificity of the antibody to the synthetic peptide epitope was tested by ELISA, Western blot of the peptide, neutralization of the antibody with excess peptide prior to Western blot analysis, and Western blot analysis of rabbit reticulocyte lysate. Immunoreactivity was visualized using an enhanced chemiluminescence method (ECL-Plus; Amersham). The band intensity was digitized (Kodak ID Image Analysis Software; Kodak) and expressed as a percentage of the contralateral control gland.

Analysis of eIF-2 α Kinase Phosphorylation Activity in Lacrimal Gland

The contralateral intact and denervated lacrimal gland fragments were solubilized by homogenizing in 200 μ l of PBS buffer (pH 7.4) containing 50 mM glycerol-2-phosphate, 1 mM PMSF, 1.5 mM EGTA, 1.0 mM DTT, 1 mM sodium vanadate, and 10 μ g/ml each of leupeptin and aprotinin, followed by centrifugation at $10,000 \times g$ for 10 min. For measurement of eIF-2 α kinase phosphorylation activity, 20 μ g of tissue protein was added to the kinase buffer (20 mM Tris-HCl (pH 7.4), 2 mM Mg-acetate, 40 mM KCl, and 2 mM DTT, 25 μ M ATP, 10 μ Ci of [γ - 32 P]-ATP (4500 Ci/mmol; ICN Biomedicals, Costa Mesa, CA, USA), and the mixture was brought to a final volume of 20 μ l. The phosphorylation activity of eIF-2 α kinase was assessed using 1 μ g eIF-2, and HRI eIF-2 α kinase was used as a positive control (gifts from Dr. J. J. Chen, Harvard-MIT Division of Health Sciences and Technology Center). The reaction was performed at 30°C for 10 min and terminated by addition of 2 $\times$ Laemmli buffer. A 10- μ l aliquot of the reaction was separated on 10% SDS-polyacrylamide gel and autoradiographed. The band intensity was digitized using Kodak 1D Image Analysis Software, and results were expressed as a percentage of the contralateral control.

Statistical Analysis

For continuously variable outcomes such as blot density, the appropriate design in the analysis of variance (ANOVA) or Student's *t* test was applied (Statistica v. 6.0; Statsoft, Inc. Tulsa, OK, USA).

RESULTS

Differential Expression of eIF-2 α Kinase

Because both contralateral intact and denervated lacrimal glands were obtained from the same animal under the same physiological conditions, differences in gene expression between the two conditions were compared using differential mRNA display (Fig. 1A).¹² The identification of differentially expressed transcripts was determined by side-to-side comparison of banding (amplicons) patterns in the control sample lane and its corresponding denervated sample lane after separation on polyacrylamide gel. Bands that were seen to be present in the control sample lane and not in the denervated sample lane were removed for further analysis. Similarly, bands that were present in the denervated sample lane and not in the control sample lane were removed. A total of 86 bands were excised from the differential mRNA display gel for PCR amplification. Of these, 44 bands gave rise to a single PCR-amplicon, and these were further evaluated by DNA sequencing and Blastn analysis. Significant numbers of nucleotide sequences were obtained from nine amplicons (Table 2). Five amplicons were found in the denervated LG (upregulated transcript), and four amplicons were associated with the contralateral intact LG (downregulated transcript). Four of the upregulated genes did not show significant homology to known genes. One of the upregulated genes (no. 4) showed some similarity to a gene for human thioredoxin-like 1. Because most of these “unknowns” are short transcripts of approximately 70 n.t., it is plausible that they may be homologues of known microRNAs (miRNAs). The miRNAs do not code for a functional protein. Instead, they encode a short (20–22 n.t.) transcript that functions as a translational regulator of the target mRNA.¹⁷ The results of sequence analysis using the miRNA Registry (www.microrna.sanger.ac.uk) showed that transcripts no. 4 and no. 5 are miRNA orthologues of several known, mature miRNAs.^{17–20} On the other hand, two of the four downregulated genes were identified as rabbit HRI-eIF-2 α kinase (no. 8) and rabbit lipophilin AL (no. 9). HRI eIF-2 α kinase, a negative regulator of protein synthesis, was considered to be an important matched transcript. HRI eIF-2 α kinase was initially identified as a band that was present in the control lane and not in the denervated lane. Because the band corresponding to HRI eIF-2 α kinase was not visibly present in the denervated lacrimal gland, it suggested that this transcript was expressed at only very low or undetectable levels in the denervated lacrimal gland. As this kinase has a pivotal

role in the control of protein synthesis, the loss (or greatly reduced expression) of this kinase could lead to higher levels of protein synthesis in the denervated gland. That is, translation would no longer be tightly controlled as in the control lacrimal gland. The 141-nucleotide sequence showed a 94% identity to HRI (Figs. 1A and 2). This sequence corresponds to nucleotides 734–875 of HRI eIF-2 α kinase.²¹

The decreased expression of HRI-eIF-2 α kinase in the lacrimal gland after sensory denervation was confirmed by Northern blot analysis (Fig. 1B), which showed a single hybridized product of approximately 3.0 kb, close to the reported 2.8–3.0 kb size of rabbit HRI eIF-2 α kinase^{21, 22} but different from the sizes reported for other eIF-2 α kinases, including mouse PKR (2.4 kb) and rat PEK (4.5 kb).^{23,24} In the current study, the level of HRI eIF-2 α kinase mRNA in the denervated lacrimal gland (Fig. 1B: lane 4) was reduced, compared with the level in lacrimal gland from the contralateral intact side (Fig. 1B: lane 5) and from normal, unoperated animals (Fig. 1B: lane 1). Moreover, the expression of eIF-2 α kinase was not altered after sympathectomy (Fig. 1B: lanes 2 and 3), suggesting that sensory denervation specifically affects the expression of HRI eIF-2 α kinase. The expression of the control gene, GAPDH, did not differ regardless of the conditions (Fig. 1C: lanes 1–5). When the densitometric results were compared with the expression of GAPDH, the expression of eIF-2 α kinase was significantly greater in all conditions (the normal unoperated and the intact contralateral control lacrimal glands), compared with the sensory denervated lacrimal gland ($p < 0.05$) (Figs. 1D and 1E). Because the role of eIF-2 α kinase is to inhibit protein synthesis, the decreased expression of this kinase in the denervated lacrimal gland suggests that protein synthesis may be under reduced regulation.

Western Blot Analysis of HRI eIF-2 α Kinases

Western blot analyses of HRI eIF-2 α kinase in the contralateral intact and the sensory denervated lacrimal gland were compared using the p74 HRI monoclonal antibody. The results showed a specific band at approximately 92 kDa in the rabbit reticulocyte lysate (RRL) positive control, as described by Chen et al.¹⁶ The levels of HRI in the denervated lacrimal gland were decreased compared with those of the contralateral intact lacrimal gland (Fig. 3, top). When these levels were expressed as percentages, HRI eIF-2 α kinase in the denervated LG was significantly decreased compared with the contralateral intact lacrimal gland ($p < 0.05$, $n = 3$) (Fig. 3, bottom). This result further suggests that sensory denervation of the lacrimal gland is accompanied by the loss of HRI, a critical control component of protein synthesis. Previously, we have shown by gel analysis of [³⁵S]methionine-labeled lacrimal proteins and by MALDI mass spectrometry of tear proteins that suggested protein synthesis may be more efficient in the denervated gland. Moreover, the tear protein profile showed that specific proteins may be preferentially synthesized in the denervated eye.⁹ These results suggest that under the condition of decreased eIF-2 α kinase expression in the denervated LG, both overall protein synthesis as well as the synthesis of specific tear proteins may be affected.

Assessment of HRI eIF-2 α Kinase Phosphorylation Activity

The phosphorylation activity of eIF-2 α kinase in the contralateral intact and denervated lacrimal glands was compared by *in vitro* measurement of eIF-2 phosphorylation. The eIF-2 is a trimeric (α , β , γ) protein complex that mediates the binding of the initiator transfer RNA (Met-tRNA_i) to the 40S ribosome. The α -subunit of eIF-2 is a specific, natural substrate of the members of the eIF-2 α kinase family, and phosphorylation of eIF-2 α leads to inhibition of protein synthesis.²⁵ The results of the *in vitro* kinase assay (Fig. 4) showed a significantly reduced phosphorylation of eIF-2 α in the denervated lacrimal gland, indicating that HRI eIF-2 α kinase phosphorylation activity is decreased as a result of sensory denervation ($p < 0.05$, $n = 3$).

DISCUSSION

The net quantity of stored secretory granules in the exocrine lacrimal gland at any one time is determined by the rate of protein synthesized, the rate of protein released by stimulation, the rate of basal protein release, and the rate of protein degradation inside the cell.^{26,27} Failure to release secretory granules may interfere with the control of protein synthesis, nascent granule formation, vesicular trafficking, and targeting.²⁸⁻³¹ Using differential mRNA display, we identified a gene, HRI eIF-2 α kinase, the expression of which was significantly reduced after trigeminal ablation as shown by Northern and Western blot analysis. Moreover, sympathetic denervation, which did not affect lacrimal function or structure,⁶ did not alter the expression of HRI eIF-2 α kinase. Finally, analysis of eIF-2 α kinase phosphorylation activity by the phosphorylation of the alpha subunit of eIF-2 also showed reduced eIF-2 α kinase phosphorylation activity in the lacrimal gland after trigeminal ablation. These results suggest that regulation of protein synthesis may be affected by the loss of trigeminal sensory fibers.

The accumulation of acinar cell granules that we observed may be due to the failure of neurotransmitter release as a result of decreased or diminished activation of the preganglionic, parasympathetic output, as demonstrated⁷ and suggested⁴ by others. However, HRI was not altered in parasympathectomized animals. The local influence of neuropeptides within the lacrimal gland from TG neurons may have a role. Sensory denervation may lead to diminished levels of substance P (SP) and calcitonin gene-related peptide (CGRP) in the gland. SP- and CGRP-immunoreactive nerve fibers are found in interlobular glandular stroma, predominantly around ducts and blood vessels.³² These neuropeptides have also been shown to modulate protein release from LG *in vitro*.³³

The initial identification of HRI in the lacrimal gland poses an intriguing question because HRI is classically known as an erythroid-specific protein.^{16,21,22} However, HRI from rat brain showed 82% similarity to HRI from erythroid cells.³⁴ HRI has also been found in mouse nonerythroid cells,³⁵ and a human HRI has been discovered with wide tissue distribution similar to that of the protein isolated from rabbit reticulocytes.³⁶ Western blot analysis positively identified HRI in the lacrimal gland, which is antigenically similar to that from erythroid cells. The reduced expression (and phosphorylation activity) of HRI in the denervated lacrimal gland suggest that mRNA translation may be less controlled and more active than in normal lacrimal gland. Moreover, the reduced expression of HRI appeared to be a specific effect of sensory denervation, as parasympathetic denervation did not appear to affect the expression of HRI.³⁷ It is possible that sensory neurons in the LG may have a role in modulating protein synthesis.

HRI is a member of the eIF-2 α kinase family that includes the dsRNA-activated protein kinase (PKR) and PKR-like endoplasmic reticulum protein kinase (PERK, also known as PEK), and the yeast GCN2.²⁵ Members of the eIF-2 α kinase family are differentially responsive, depending on the nature of the environmental conditions (heat shock, Ca²⁺ release from endoplasmic reticulum, viral infection, and amino acid deprivation). These eIF-2 α kinases phosphorylate the same alpha subunit of eIF-2, which leads to inhibition of protein synthesis at the level of translational initiation. HRI is a cAMP-independent protein kinase activated by heme-deficiency and oxidative stress induced by arsenite, heat shock, and osmotic stress.^{16,38} Given the functional role of HRI in mediating the stress response, the decreased expression of HRI suggests a reduced capacity of the sensory denervated LG to respond to additional cytoplasmic perturbations and, consequently, regulation of protein synthesis.

In summary, our results suggest that the loss of sensory TG neurons subserving the cornea affects secretory protein synthesis in the LG by modulating the initiation step of protein synthesis. Previously, we have shown that protein synthesis appeared to be more efficient and

at a higher level in the denervated lacrimal gland (under the condition of decreased eIF-2 α kinase expression). Furthermore, analysis of tear proteins found differences in the tear protein profile suggesting specific protein may be differentially synthesized.⁹ The decreased expression of HRI may be from decreased activation of neurotransmitter membrane receptors or from alteration of vesicular trafficking. Moreover, other eIF-2 α kinases and regulatory translation factors such as eIF-4 and eEF-2 may also be affected.³⁵ The expression of other differential mRNA display transcripts, including the identification of several miRNAs, will be explored in future studies. The decreased expression of HRI eIF-2 α kinase in the sensory denervated LG may potentially affect the regulation of mRNA translation, a dysfunction that may lead to increased protein synthesis and cellular demands for dealing with increased load in lysosomal degradation pathways. Moreover, the reduced inhibitory control of protein synthesis may stimulate the translation of repressed messages that are involved in the stress response. The balance among protein synthesis, secretory granule formation and maturation, and secretion may be under both sensory and parasympathetic regulation.

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FIGURE 1.

Identification and measurement of HRI eIF-2 α kinase expression in control and denervated LG. (A) Differential mRNA display of a 320-bp DNA fragment found in the contralateral intact LG but not in the denervated LG. The DNA fragment was sequenced and found to have a 94% sequence identity to HRI eIF-2 α kinase. (B) Northern blot analysis of HRI showed the radiolabeled fragments hybridized at approximately 3.0 kb. Expression of HRI was similar for normal unoperated LG (lane 1), intact LG contralateral to the sympathectomized gland (lane 2), sympathectomized LG (lane 3), and intact LG contralateral to the sensory denervated gland (lane 5). Only the sensory-denervated gland (lane 4) showed reduced expression of mRNA for HRI. (C) Northern blot analysis of the stripped blot probed with a PCR-generated GAPDH using gene-specific primers. The probes hybridized at approximately 1.2 kb, and GAPDH expression was similar in all conditions. (D) Histograms of eIF-2 α kinase and (E) GAPDH expression in normal unoperated (Norm), contralateral intact of sympathectomized (C-Sym), sympathectomized (D-Sym), sensory denervated (D-Sen), and contralateral intact of sensory denervated LG (C-Sen). Expression of eIF-2 α kinase in all conditions was significantly greater than expression in denervated LG ($P < 0.05$, $n = 3$). No significant differences were observed in the expression of GAPDH. Data are means $\pm$ SEM.

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1  AAAACAGATTTCGCATGACGGTATTACGAGAAGTGAAAGTGCC*GGCGGGCCTCCAGCACCC
  ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
734 AAAACAGATT*GCATGAAGGTATTACGAGAAGTGAAAGTGC*GGCGGGCCTCCAGCACCC

62  TAAATATCGTAGGCTATCACACCGCGTGGATAGAGCATGTCCACGTTCAAG----
  ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
793 TAA*TATCGTAGGCTATCACACCGCGTGGATAGAGCATGTCCACGTTCAAGCAGA

122 CAGAGTTCGATTAGTTGCCTT 141
  ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| ||| |||
853 CAGAGTTCGATTAGTTGCCTT 875

```

FIGURE 2.

Alignment of the DNA sequence of the differential mRNA display fragment (141 n.t.) to the rabbit HRI eIF-2 α kinase.¹⁷ The 141 nucleotide sequences showed 94% identity to the rabbit HRI (734–875 n.t.). Sequences that were not obtainable from DNA sequencing are indicated by (–). Gaps in the rabbit HRI eIF-2 α kinase are indicated by (*).

FIGURE 3.

Sensory denervation produced a decrease in the levels of HRI in the LG after 7 days. Top: Western blots of anti-p74 HRI per μg protein showed the level of HRI was lower in the denervated (DEN; 50 μg) LG than in the contralateral (CTLA; 50 μg) intact control. +, positive control, 1 μg (rabbit reticulocyte lysate). Bottom: Expression of HRI determined with the anti-p74 antibody was significantly higher (*) in the contralateral intact LG than in the denervated LG ($p < 0.05$, $n = 3$). The value for the DEN group is expressed as a percentage of the CTLA value. Data are means $\pm$ SEM.

FIGURE 4.

Phosphorylation activity of eIF-2 α kinase per μ g protein was decreased in the denervated LG after 7 days. Top: Phosphorylation activity of eIF-2 α kinase in the contralateral intact (CTLA) and denervated (DEN) LG was determined by an *in vitro* kinase assay using the eIF-2 α protein and separation on a 10% SDS-polyacrylamide gel. -, eIF-2 α alone; +, HRI + eIF-2 α . Bottom: Histogram shows a significant decrease (*) in the level of phosphorylated eIF-2 α in the denervated LG compared with control ($P < 0.05$, $n = 3$). Data are means $\pm$ SEM.

TABLE 1
Primers used for differential mRNA display

	Upstream Primers	Downstream Primers
AP-1	5'-AGCCTGTGTC-3'	
AP-2	5'-CAAGCGAGGT-3'	
AP-3	5'-AACGCGCAAC-3'	5'-T ₁₂ CA-3'
AP-4	5'-GTGGAAGCGT-3'	5'-T ₁₂ GG-3'
AP-5	5'-GGAAGCAGCT-3'	5'-T ₁₂ CT-3'
AP-6	5'-CAGTGAGCGT-3'	5'-T ₁₂ GC-3'
AP-7	5'-GAGCTATGGC-3'	

Arbitrary primers (AP-1 to AP-7) were selected from Sokolov and Prockop¹³. Downstream primers were selected from Liang and Pardee¹². This gives 28 primer pair combinations for differential mRNA display.

TABLE 2

Differential mRNA display analysis of upregulated genes in sensory denervated lacrimal glands and downregulated genes in contralateral intact lacrimal glands

Band #	Change	% Identity	Gene
1	Up	?	Unknown, (159 n.t.). ttctcgcttgcttgctatcagcgttagcgcacatgctctttgacggtatgtgcagcctgtgttcctt tatcttggctctggctctgtgctgacgtacttactgctacacctgtccaggactaatctctgtctctc gttgcctggt
2	Up	?	Unknown, (103 n.t.). tagtgcacttgattagcaggaagtcagagggtgcaatcattcagagaatcaatattgtagctctttatac ggattgacataagctgicattccgag
3	Up	38/40	Similar to human thioredoxin-like 1 (gi 21754089), (71 n.t.). cacttggaaagaccatttgcaatcagactactacgctccctggtacccgttgattcttcgcagagcttcattgtt
4	Up	?	miRNA-181b ortholog, (70 n.t.). gcagcagctgctgacccaatagagaacgattgctggtgagcagctgacatcattactgtcgggtcagcgc
5	Up	?	hsa-miR-369-5p ortholog, (87n.t.). taagcattctcagagctgacgtagtagacgtgacgtacagatcgtgcatgctatgcatgattcgcatatcac gtcagatctcatg
6	Down	?	Unknown, (63 n.t.). gttctcttacttacttagagatccattgacggtagacatttccaaagagctagcagctca
7	Down	53/56	Similar to Hs532786 (93 n.t.). ttcactaaactttcattggcgctaattggaattatggaaagggtgattggattctttcccttttgggaagtaaa ctctcagtaactat
8	Down	137/145, 94%	Rabbit HRI eIF-2 α kinase, (141 n.t.), accession # M69035.
9	Down	80/89	Rabbit lacrimal gland lipophilin AL, (98 n.t.), gi 11993591. gaaaatgcaagtgcaagggaatgcatggttgagattgcaaaaggaaaagagcgttaattgcagcagtgct gccaaatagtgaaagaatgtgcttgga

The *Change* column indicates whether the gene is upregulated or downregulated. The % *Identity* column indicates the number of nucleotides (n.t.) of the input sequence that matches the sequences of known genes in the National Center for Biotechnology Information database. Sequences of the input genes that are similar to the known genes are underlined. Nucleotide sequences of HRI eIF-2 α kinase are displayed in Figure 2.