Dynein light chain interacts with NRF-1 and EWG, structurally and functionally related transcription factors from humans and Drosophila

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SUMMARY

Nuclear respiratory factor-1 is a transcriptional activator that has been implicated in the nuclear control of respiratory chain expression. Yeast two-hybrid screens were performed to identify proteins that physically interact with nuclear respiratory factor-1. Saturation screening of both mouse embryo and mouse testis libraries yielded 14 independent clones, all of which represented two different isoforms of dynein light chain. In addition to using the two-hybrid method, the specificity of the nuclear respiratory factor-1/dynein light chain interaction was established by chemical crosslinking of the purified native proteins and by co-immunoprecipitation of nuclear respiratory factor-1 and dynein light chain from mammalian cells. Both two-hybrid and chemical crosslinking assays demonstrated that binding of dynein light chain required the first 26 amino acids of nuclear respiratory factor-1. Although dynein light chain is associated with dynein, a cytoplasmic motor molecule, immunolocalizations showed substantial nuclear staining using several different anti-dynein light chain antibodies. Moreover, fluorescence overlays of confocal images established that nuclear respiratory factor-1 and dynein light chain displayed a very similar nuclear staining pattern. The significance of the nuclear respiratory factor-1/dynein light chain interaction was investigated further by determining whether a similar interaction was conserved between dynein light chain and the erect wing gene product of Drosophila, a protein related to nuclear respiratory factor-1 through its DNA binding domain. Here, we establish that the erect wing gene product can bind and trans-activate transcription through authentic nuclear respiratory factor-1 binding sites. Moreover, the erect wing gene product, like nuclear respiratory factor-1, interacted specifically with dynein light chain both in vitro and in transfected cells. Thus, the interaction with dynein light chain is conserved between transcription factors that are structurally and functionally similar between humans and Drosophila.

Key words: Nuclear respiratory factor, Yeast two-hybrid, Protein-protein interaction, Erect wing, Nuclear DLC

INTRODUCTION

The dyneins are multisubunit proteins that associate with microtubules and function as molecular motors (reviewed by Vallee and Sheetz, 1996). In lower eukaryotes, the axonemal dyneins are necessary for the motility of cilia and flagella (King and Patel-King, 1995). In non-ciliated, higher eukaryotes, microtubule-associated cytoplasmic dyneins have been implicated in a number of cellular functions including cytoplasmic organelle movement, retrograde transport in axons, nuclear migration as well as in positioning and possibly assembly of the mitotic spindle (reviewed by Vallee and Sheetz, 1996). The dyneins are comprised of subunits ranging in mass from 9 to over 500 kDa. The largest of these subunits, the heavy chains, are involved in force production whereas the others are defined as accessory subunits based on their association with the dynein complex. The functions of these accessory subunits are not known although the intermediate chains have been implicated in targeting dynein molecules to cellular binding sites. The smallest dynein subunits, the 9 kDa light chains (DLC), are thought to be associated with the intermediate chains in both axonemal and cytoplasmic dyneins (King and Patel-King, 1995) but their significance to dynein function has not been established. Interestingly, three distinct pools of DLC exist within the cell and the predominant form was not associated with microtubules (Benashski et al., 1997).

Two-hybrid screenings have revealed that DLC interacts with several unrelated cellular proteins suggesting that it may have multiple functions. DLC has been identified as an inhibitor of neuronal nitric oxide synthase, a key enzyme in the regulation of nitric oxide biosynthesis (Jaffrey and Snyder, 1996). In this case, DLC inhibited nitric oxide synthase by interfering with subunit dimerization, which is a requirement for enzymatic activity. DLC was also found to interact with IκBα, a molecule that inhibits the NF-κB/Rel family of transcription factors by promoting their cytoplasmic retention (Crepieux et al., 1997). The amino terminus of IκBα helps promote its regulated proteolysis thus allowing the nuclear translocation of NF-κB. DLC interacted with the amino-terminal domain of IκBα and was localized by subcellular
fractionation and by immunofluorescence labeling to both cytoplasmic and nuclear compartments. The potential involvement of DLC in the regulation of apoptosis was suggested by its interaction with Bim, a proapoptotic member of the Bcl-2 family (Puthalakath et al., 1999). Under normal conditions Bim and DLC formed a complex that was sequestered in the cytoplasm by association with the microtubule-associated dynein. Apoptotic signals prompted the dissociation of the Bim/DLC complex from microtubules and its translocation to the mitochondria to promote the apoptotic activity of Bcl-2. A multifunctional role for DLC is also consistent with the observation that DLC has been identified as a subunit of myosin V, an actin based motor molecule (Benashski et al., 1997).

In this work, we establish that DLC binds the amino-terminal domain of NRF-1 (nuclear respiratory factor 1), a nuclear transcription factor that acts on genes involved in establishing and maintaining mitochondrial respiratory function (reviewed by Scarpulla, 1997). NRF-1 was initially discovered as an activator of cytochrome c expression and was subsequently found to act on many other nuclear genes encoding respiratory subunits. In addition, NRF-1 has been implicated in the expression of nucleus-encoded components of the mitochondrial DNA transcription and replication machinery as well as in the expression of the rate-limiting heme biosynthetic enzyme (Braidotti et al., 1993; Virbasius and Scarpulla, 1994). These findings suggested that NRF-1 is involved in coordinating nuclear and mitochondrial gene expression in meeting cellular energy demands.

NRF-1 is one of three regulatory factors that are related by a common DNA binding domain. P3a2, a NRF-1 relative in sea urchins, is required for gene expression in the aboral ectoderm and the skeletogenic mesenchyme, two of the five territories established in developing sea urchin embryos (Calzone et al., 1991; Hoog et al., 1991). The ecdysozoan gene product EWG of Drosophila is an NRF-1 relative that is expressed in the developing nervous system and is required for proper neuromuscular development (Desimone and White, 1993). EWG has been localized to the nucleus but its DNA binding and transcriptional activities have not been demonstrated nor have target genes been identified. Interestingly, mutants in EWG and DLC display very similar phenotypes and the two proteins were localized to identical subcellular domains (Desimone and White, 1993; Dick et al., 1996; Phillis et al., 1996). The results suggest that DLC has a nuclear function that is conserved between humans and Drosophila.

MATERIALS AND METHODS

Plasmid constructs

'Bait' plasmids used in the yeast two-hybrid system were derivatives of pBTM116, a yeast expression vector that carries the TRP1 gene (Bartel and Fields, 1995). In this vector, the full-length LexA gene is fused to the amino terminus of a desired coding region. pBTM116NRF-1/1-503 contains the entire NRF-1 coding region (503 amino acids) and was constructed by adapting and ligating the 221-bp NcoI/PstI and 1322-bp PstI/BamHI fragments of pSG5NRF-1/119-1662 (Virbasius et al., 1993) into Smal/BamHI-cut pBTM116. pBTM116NRF-1/1-304 lacks sequences encoding the carboxy-terminal NRF-1 activation domain and was constructed using the 690-bp PstI/BamHI fragment from pSG5NRF-1/1-1030 (Virbasius et al., 1993). pBTM116NRF-1/304-477 expresses only the activation domain and was made by ligating the BamHI fragment from pGAL4/NRF-1 (304-477) (Gugneja et al., 1996) into BamHI linearized pBTM116. The orientation was verified by sequencing. pBTM116NRF-1/1-263 was derived using the PstI/BamHI insert from pSG5NRF-1/1-908 (Virbasius et al., 1993). pBTM116NRF-1/1-74 was derived from pBTM116NRF-1/1-503 by releasing the region 3' from the PstI site by PstI digestion and re-ligating. pBTM116NRF-1/76-304 was made by cloning the insert from pSG5NRF-1/348-1662 (Virbasius et al., 1993) into NcoI/EcoRI-digested pBTM116NRF-1/1-304. Similarly, pBTM116NRF-1/42-304 was made by cloning the NcoI/EcoRI insert from pSG5NRF-1/42-304. The resulting PCR amplification product was digested with NcoI and EcoRI and the 493 bp fragment was then ligated into pBTM116NRF-1/1-304 that was linearized with NcoI and EcoRI to release a 574-bp NRF-1 fragment.

pBTM116LamIN and pBTM116AKR7 (gifts from Thomas Donahue, Indiana University) were used as negative controls for verifying the yeast two-hybrid positives.

The pSG5-based plasmids were used for expression of NRF-1, EWG and DLC in COS and C6 glial cells. Construction of pSG5NRF-1/142-503 has been described previously (Gugneja and Scarpulla, 1997). pSG5EWG was made by taking the EcoRI fragment from pHsp-SC3/EWG (Desimone and White, 1993) and cloning it into the EcoRI site of pSG5. The orientation was verified through restriction mapping. pSG5DLC was made by PCR amplification of the mouse-a DLC coding region from one of the 10-day mouse embryo library two-hybrid positives. Forward DLC-R1F (5' CCGAATCTATGGCGACCGA 3') and reverse DLC-BamR (5' AGTGGATTCCCCATACAGTAG 3') primers contained EcoRI and BamHI sites, respectively. The PCR amplification product was digested with EcoRI and BamHI and cloned into the same sites in pSG5. pSG5NRF-1/3XHA has been described (Gugneja and Scarpulla, 1997). The pET3d-based plasmids were used for bacterial expression of recombinant proteins. The DLC coding region in pET3d/DLC 6His was derived by PCR amplification with primers DLC-Nco-F (5' TAACCATGGGCGACCCGA 3') and DLC-Acc-R (5' TCTTGTTACCTTACAGTGAG 3'). The resulting PCR amplification product was digested with NcoI and Acc65I and cloned into pET3dNRF-1/6His (Gugneja and Scarpulla, 1997) following release of the NRF-1 coding region by digestion with NcoI and Acc65I. This places the 6His tag at the carboxy terminus of DLC.

The Drosophila expression vector pNPC5c (Urn, 1995), utilizes the Drosophila actin 5c gene promoter to drive expression in transfected SL2 cells. To make pNPC5cNRF-1, the 5' portion of the NRF-1 coding region was excised from pSG5NRF-1/119-1662 with EcoRI and PstI and cloned through a pBluescript (Stratagene) intermediate to add an EcoRV site to the 5' EcoRI end. The 3' portion of the NRF-1 coding region was excised from pET3dNRF-1/6His with PstI and Acc65I, and together with the EcoRV/PstI 5' NRF-1 fragment, cloned into pNPC5c linearized with EcoRV and Acc65I.
pPac5cEWG was made by excising the BamHI/SacI EWG fragment from pG5SEWG and cloning it into pPac5c.

 Luciferase reporter plasmids containing the rat cytochrome c promoter (pGL3RC4-326) was used for expression in SL2 cell transfections, pGL3RC4-326 was constructed by digesting pRC4CAT/326 (Evans and Scarpulla, 1988) with BstII filling in the end with Klenow DNA polymerase and subsequently digesting it with XhoI. The resulting fragment containing the promoter, 5′-UTR and first intron and was cloned into pGL3 basic vector (Promega) that had been cut with HindIII, blunt-ended with Klenow and digested with XhoI.

 Yeast two-hybrid screen

Saccharomyces cerevisiae L40 (MATa his3A200 trp1A-901 leu2A-3,112 ade2A LYS2::(lexAop)-HIS3 URA3::(lexAop)-lacZ GAL4 gal80 L40pBTM116NRF-1/1-304, 2; pBTM116Sui1p, 3; pBTM116 as indicated by the numbered sectors in each panel. The growth medium or β-gal staining is labeled below each panel.

Interaction of dynein light chain with NRF-1

Production of recombinant DLC and anti-DLC sera

E. coli strain BL21 (DE3) pLysS was transformed with pET3dDLC6His and the transformants plated on LB with 25 µg/ml chloramphenicol and 100 µg/ml ampicillin. A single colony was grown and protein induction was carried out as described (Sambrook et al., 1989; Gugneja and Scarpulla, 1997). Large-scale production of DLC/6His was performed in 200 ml LB medium at 28°C for 4 hours with isopropyl-β-D-thiogalacto-pyranoside at 0.4 mM. Recombinant DLC remained in the supernatant and did not precipitate with ammonium sulfate. Therefore, the supernatant was diluted to a final salt concentration of 0.85 M with water and then mixed with 5 ml of washed Qiagen NTA resin for 12 hours at 4°C. The protein-bound resin was centrifuged at 2,000 g for 5 minutes, suspended in TM buffer (25 mM Tris-HCl, pH 7.9, 6.25 mM MgCl₂, 10% glycerol, protease inhibitor cocktail) and loaded onto a Bio-Rad EconoColumn. The column was washed with 5 bed-volumes of TM buffer, 5 bed-volumes of TM buffer (pH 6) and the bound rDLC/6His eluted from the column with a linear gradient of 0-0.5 M imidazole in TM buffer (pH 6). The 4 ml fractions containing rDLC/6His were combined, concentrated in an Amicon Stirred Cell Concentrator, and dialyzed against 25 mM Tris (pH 8), 100 mM EDTA, 0.1% NP40, 10% glycerol and flash-frozen in aliquots. Estimated purity of the rDLC/6His was 99%. Rabbit polyclonal anti-rDLC/6His serum was produced by Harlan Bioproducts for Science, Inc. (Madison WI).
rDLC/6His was conjugated to keyhole limpet hemocyanin and 0.5 mg was injected once every 3 weeks for 90 days into two different New Zealand white rabbits.

Gluteraldehyde crosslinking
rNRF-1 (Gugneja and Scarpulla, 1997) and rDLC (10 nmoles each) were mixed in 9 µl PBS (phosphate buffered saline, Sigma, St Louis MO). After incubation at room temperature for 15 minutes the crosslinking reaction was initiated by the addition of 1 µl of 0.05% glutaraldehyde in PBS. Following incubation at room temperature for 1 hour, the reaction was stopped by the addition of 2 µl of 1 M glycine. Samples were electrophoresed on 4-20% linear gradient SDS-PAGE (polyacrylamide gel electrophoresis) gels and transferred to PVDF for immunoblotting as described previously (Gugneja and Scarpulla, 1997).

Co-immunoprecipitations
Co-immunoprecipitations of NRF-1 or EWG with DLC were carried out using mixtures of recombinant proteins, [35S]-labeled proteins or whole cell extracts from cells expressing the various proteins. Recombinant NRF-1 and DLC were co-immunoprecipitated by mixing 300 ng of each protein in 500 µl of 50 mM Tris-HCL, pH 8.0, 150 mM NaCl, 1% NP-40, protease inhibitor cocktail at room temperature for 15 minutes. NRF-1 and DLC were co-immunoprecipitated from transfected COS cell extracts prepared by scraping ice-cold, PBS washed cells into a chilled 1.5 ml Eppendorf tube. The cells were briefly centrifuged and the supernatant discarded. The pellet was resuspended in 500 µl of lysis buffer, incubated on ice for 15 minutes and sonicated for 3 seconds on the lowest setting with a Branson Sonifier 450 fitted with a microtip. The lysates were vortexed for 15 seconds, incubated for 5 minutes on ice and cleared a Branson Sonifier 450 fitted with a microtip. The lysates were vortexed for 15 seconds, incubated for 5 minutes on ice and cleared by centrifugation at 20,000 g for 5 minutes. rDLC (9 µg) was also co-immunoprecipitated in vitro with [35S]EWG, [35S]NRF-1, or [35S]NRF-1/42-503, which were [35S]methionine-labeled by in vitro translation in a wheat germ extract (Promega) according to the manufacturer’s specifications. In all cases, the appropriate anti-serum was added to each protein mixture and the solutions mixed at 4°C for 1 hour (recombinant proteins) or 3 hour. Lysis buffer-washed, Protein A agarose beads were then added and mixed for 1 hour (recombinant proteins) or overnight (whole cell extracts or in vitro labeled proteins) at 4°C. The resin was pelleted at 500 g, washed three times in 1 ml lysis buffer and suspended in 20 µl of lysis buffer. After adjusting to 1× with 2× SDS loading buffer (0.125 M Tris-HCL, pH 6.8, 4% SDS, 1.43 M β-mercaptoethanol, 20% glycerol), the samples were then run on 15% SDS-PAGE gels and transferred to PVDF membrane for immunoblotting (Gugneja and Scarpulla, 1997).

Polyclonal antiserum raised against EWG was a gift from Kalpana Babco. Rabbit anti-DLC sera were diluted 1:200 in PBS for use as a primary antibody and goat anti-rabbit FITC-conjugated IgG was diluted 1:150 in PBS as a secondary antibody. Antibody incubations were for 1 hour followed by 3, 5 minute washes in PBS. Rabbit anti-DLC sera were diluted 1:200 in PBS for use as a primary antibody and goat anti-rabbit FITC-conjugated IgG was diluted 1:150 in PBS as a secondary antibody. Antibody incubations were for 1 hour followed by 3, 5 minute washes in PBS. After the final wash, the coverslip was mounted onto a glass slide with a glycerol/PBS mounting medium containing antioxidant (50 mM sodium bicarbonate, pH 8.75 mM NaCl, 1 mg/ml p-phenylenediamine, 80% glycerol).

For confocal immunofluorescence microscopy, cells grown on a 10 cm dish were transfected using CaPO4 with 5 µg pSG5/NRF1-3xHA (Gugneja and Scarpulla, 1997) and 15 µg pBluescript (Stratagene) as carrier. Immediately following transfection, cells were re-plated on 4-well Lab-Tek II chamber slides (Nalge-Nunc). The medium was discarded 24 hours following inoculation and cells were rapidly chilled on ice-cold metal block and fixed in ice-cold 4% paraformaldehyde in PBS over night at +4°C. Next, slides were incubated for 30 minutes at room temperature in 1% BSA/0.2% Triton X-100 in PBS, which was also used in all following steps. For immunolabeling, samples were incubated for 60 minutes with primary antibodies, washed 3 times 5 minutes, incubated 60 minutes with secondary antibodies, washed as above, rinsed in de-ionized water, mounted in Mount-Quick (Electron Microscopy Services (Mt Washington, PA) and viewed in a Zeiss LSM510 confocal laser scanning microscope. Primary antibodies used were rabbit anti-DLC (diluted 1:50) and mouse anti-HA (diluted 1:200; Babco). Secondary antibodies used were goat anti-mouse IgG conjugated to Rhodamine Red X (diluted 1:200; Jackson Immunoresearch) and goat anti-rabbit IgG conjugated to Alexa Fluor 488 (diluted 1:100; Molecular Probes).

Gel electrophoresis shift assay Nuclear electromobility shift assays were prepared as described previously (Andrews and Faller, 1991). The labeling of oligonucleotide RC4-172/-147 and the gel electromobility shift assay were performed as described previously (Virbasius et al., 1993). The serum response element (SRE) (5’ ATATCCAAAATATGCCTTGAGTC 3’) (Lee et al., 1991) was used as a non-specific competitor oligonucleotide. Competitor oligonucleotides were present in 100-fold molar excess. The supershifting antibody was added 10 minutes after the shift reaction components were mixed and allowed to incubate for an additional 5 minutes before loading onto the gel.

Transfections and reporter assays COS cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% iron-supplemented calf serum (Hyclone Laboratories, Logan UT) and 1% Pen/Strep. solution (Sigma, St Louis MO). Schneider SL2 cells were purchased from the American Type Tissue Collection at passage 518 and grown at 25°C in Schneider’s insect medium (Sigma, St Louis MO) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan UT) as described (Cherbas and Cherbas, 1998). Transfection of both COS and SL2 cells was done by calcium/phosphate (Ausubel et al., 1990).
RESULTS

A specific interaction between NRF-1 and DLC by two-hybrid screening

The vector pBTM116 was used to express NRF-1 as ‘bait’ for two-hybrid screening. In this vector the full-length LexA protein is used to promote efficient dimerization. Moreover, expression is driven by a truncated ADH1 promoter to facilitate low-level expression of the LexA/NRF-1 fusion and thereby minimize non-specific interactions that may result from high-level expression. Expression of a fusion containing full-length NRF-1 (NRF-1/1-503) from this vector promoted growth on –His medium and produced β-galactosidase (Fig. 1A). This was also the case for NRF-1/304-477 indicating that the activation domain of NRF-1 is functional in yeast cells. Because of this, the full-length construct was unsuitable for two-hybrid screening. Deletion of the activation domain in NRF-1/1-304 eliminated both growth on –His and β-galactosidase activity. Therefore, NRF-1/1-304 was used for screening.

A 9.5/10.5 day mouse embryo library and a mouse testis library were screened. Ten colonies from the mouse embryo library and 28 colonies from the mouse testis library were His prototrophs in the presence of 3-AT and positive for β-galactosidase. In order to eliminate false positives the isolates were further tested using negative control ‘bait’ plasmids including pBTM116Lamin, pBTM116Sui1p and pBTM116AKR7 (Fig. 1B). Of the 38 original isolates, 14 retained His prototrophy and β-galactosidase expression upon retransformation. These all were positive for NRF-1 interaction but were negative for interaction with the negative controls and were therefore taken as true positives. DNA sequencing demonstrated that they all encoded one of two variants of mouse DLC designated as mouse-a and mouse-b (Fig. 2). Interestingly, the mouse-b isoform had not been previously described. All of the mouse-b clones were isolated from the testis library raising the possibility that this variant may be testis-specific.

Fig. 3. Specificity and mapping of the NRF-1/DLC interaction by glutaraldehyde crosslinking. (A) Glutaraldehyde concentration dependence on the crosslinking of NRF-1 and DLC. Equimolar amounts of purified recombinant DLC and NRF-1 were mixed and glutaraldehyde added to final concentrations ranging from 0 to 0.02% (v/v). The crosslinked products were electrophoresed on a 4-20% linear gradient SDS-PAGE gel and transferred to PVDF membrane for immunoblotting with anti-DLC serum. (B) Purified recombinant NRF-1 (1-503) or its mutated derivatives (1-330 or 42-503) were added alone (–) or mixed with equimolar amounts of purified recombinant DLC (+) as indicated above each lane. Glutaraldehyde was either absent (–) or added to final concentration of 0.005% (v/v) (+). Binding reactions were electrophoresed on 4-20% linear gradient SDS-PAGE gels. The gel was subjected to immunoblotting using anti-NRF-1 serum as the primary antibody. (C) An identical gel was subjected to immunoblotting using anti-DLC serum as the primary antibody. The positions of molecular mass standards are shown at the left in all three panels.
Interaction with DLC requires the first 26 amino-terminal residues of NRF-1

A glutaraldehyde crosslinking assay was developed to confirm that DLC could interact with full-length native NRF-1 in vitro. Equimolar amounts of NRF-1 and DLC were mixed and treated with varying concentrations of glutaraldehyde. A high molecular mass species of approximately 175 kDa was detected at low concentrations of glutaraldehyde (0.003%; Fig. 3A). This species increased with increasing concentrations of crosslinker as the 9 kDa DLC was depleted from the crosslinking reaction. The formation of the high molecular mass complex was NRF-1-dependent and contained NRF-1 as revealed by immunoblotting with anti-NRF-1 serum (Fig. 3B and C, lanes 1, 2, 7, and 8). Although this species was similar in size to the previously defined NRF-1 homodimer (Gugneja and Scarpulla, 1997) (Fig. 3B, lanes 1, 2) it reacted specifically with anti-DLC serum only when NRF-1 was present in the binding reaction. Interestingly, two additional crosslinked species of approximately 19 and 27 kDa, which corresponded in mass to DLC dimer and trimer, were also formed (Fig. 3A and C). These did not require the presence of NRF-1 and reacted only with anti-DLC serum (Fig. 3B and 3C, lanes 7-10). In addition, only the 9 kDa DLC monomer was depleted with increasing glutaraldehyde concentration. These results confirm the interaction between NRF-1 and DLC detected by two-hybrid screening and establish that the purified full-length native proteins can form a specific complex.

The crosslinking assay was also used to map the domain on NRF-1 required for binding to DLC. A carboxy-terminal deletion of NRF-1 (NRF-1/1-330) had no effect on the formation of the NRF-1/DLC complex (Fig. 3B and C, lanes 3, 4, 11, and 12). By contrast, an amino-terminal deletion to residue 42 (NRF-1/1-42-304) completely eliminated the NRF-1/DLC complex (lanes 5, 6, 13, and 14). The requirement for an intact amino-terminal domain was confirmed by two-hybrid assay. Carboxy-terminal deletions of NRF-1, including a severe truncation of all but the first 74 amino acids, were positive for interaction with DLC in the two-hybrid assay (Fig. 4). By contrast, all amino-terminal deletions of NRF-1 were negative including one where only the amino-terminal 26 amino acids were removed. Thus, the amino terminus of NRF-1 is the target for specific recognition by DLC.

Interaction and nuclear localization of NRF-1 and DLC in mammalian cells

DLC was originally identified as a subunit of the cytoplasmic motor molecule dynein (King et al., 1996) whereas NRF-1 is a transcription factor localized to the nucleus (Gugneja et al., 1996). Thus, it was important to determine whether these two

![Fig. 4. Mapping of the DLC interaction domain to the 26 amino-terminal residues of NRF-1. Yeast cells carrying a DLC-expressing plasmid were transformed individually with 1; pBTM116NRF-1/1-304, 2; pBTM116NRF-1/1-263, 3; pBTM116NRF-1/1-74, 4; pBTM116NRF-1/76-304, 5; pBTM116NRF-1/42-304, or 6; pBTM116NRF-1/27-304 as indicated by the numbered sectors in each panel. The growth medium or β-gal staining is labeled below each panel.](image)

![Fig. 5. Immuno-localization of DLC to the nucleus of C6 glioma cells. The subcellular location of DLC in formaldehyde-fixed C6 glioma cells was visualized by immunofluorescence using four different rabbit anti-DLC serum preparations. (A) anti-DLC serum #76, (B) anti-DLC serum #77, (C) anti-DLC serum R4058, (D) blot-purified anti-DLC serum R4058. (E) The specificity of each anti-DLC serum was assayed by immunoblotting. Approximately 100 µg of C6 glioma whole cell extract was run on a 4-20% acrylamide linear gradient gel and transferred to PVDF membrane. Blots were probed with anti-DLC serum #76 (lane 1), anti-DLC serum #77 (lane 2), anti-DLC serum R4058 (lane 3) and blot-purified anti-DLC serum R4058 (lane 4). The positions of molecular mass standards are shown at the left.](image)
molecules are present in the same subcellular compartment and whether they can interact when expressed in mammalian cells. The subcellular location of DLC was determined by immunofluorescence staining using three different antibodies (Fig. 5). Two of these were raised to purified recombinant mouse DLC (#76 and #77, Fig. 5A and B) whereas the third was raised to recombinant *Chlamydomonas* DLC (King et al., 1996) (R4058, Fig. 5C and D). Although both nuclear and cytoplasmic fluorescence was observed using each of the antibodies, the signal was most intense in the nucleus. The nuclear fluorescence did not result from a contaminating antibody that cross-reacted with a nuclear antigen. The enhanced nuclear staining was preserved upon blot purification (King et al., 1996) of the anti-*Chlamydomonas* DLC antibody (Fig. 5D). In addition, immunoblotting using whole cell extracts demonstrated intense staining of DLC using all four antibody preparations with little or no cross reactivity with other cellular antigens (Fig. 5E). The very minor cross-reacting species observed upon over exposure of the immunoblot were not common to the different antibody preparations. These results establish that a substantial fraction of DLC is localized to the nucleus.

A fluorescence overlay experiment was performed using confocal laser scanning microscopy to confirm that NRF-1 and DLC share a nuclear location. Cells were transfected with pSG5NRF-1/3XHA and a monoclonal antibody directed against HA was used to detect the HA-tagged NRF-1 in transfected cells. The results show nuclear fluorescence (green) in both transfected and untransfected cells using anti-DLC antibody (Fig. 6A,D,G). By contrast, using anti-HA antibody, exclusively nuclear fluorescence (red) was observed only in transfected cells (Fig. 6B,E,H). Merging of the fluorescent images confirms the nuclear localization of both proteins (Fig. 6C,F,I). Although the NRF-1 and DLC staining patterns were not entirely superimposed, the nuclei gave the yellow color expected of co-localization. In addition, the most homogeneous co-localization pattern was observed in transfected cells expressing lower levels of NRF-1 (compare cells in Fig. 6E and F) suggesting that the nuclear co-localization was not dependent on high levels of NRF-1 expression.

It was important to confirm that NRF-1 and DLC can interact when expressed in mammalian cells. Conditions for co-immunoprecipitation were worked out by mixing recombinant proteins in vitro, immunoprecipitating using anti-NRF-1 serum and visualizing the presence of DLC in precipitates by immunoblotting. The results show specific co-precipitation of DLC with NRF-1 when anti-NRF-1 serum was used as the precipitating antibody (Fig. 7A, lane 1). No DLC was detected in precipitates when anti-NRF-2β1 serum was used as a negative control (lane 2) or when DLC was omitted from reaction mixtures (lane 3). NRF-2β1 is structurally unrelated to NRF-1 and does not bind DLC (not shown). Co-immunoprecipitations were also carried out using whole-cell extracts from cells transfected with DLC and NRF-1 expression plasmids. DLC was immunoprecipitated using anti-NRF-1 serum (Fig. 7B, lane 1) but not with the control anti-NRF-2β1 serum (lane 2). These results support the conclusion that NRF-1 and DLC can interact when expressed in mammalian cells.

### Interaction of DLC with EWG, a NRF-1-related protein in *Drosophila*

The erect wing gene product, EWG of *Drosophila*, is required for proper neuromuscular development and is closely related to NRF-1 through its DNA binding domain (Desimone and White, 1993). As discussed below, disruption of EWG or DLC loci results in nearly identical phenotypes. Moreover, the two proteins exhibit a nearly identical expression pattern in *Drosophila* embryos (Desimone and White, 1993; Desimone et al., 1995; Dick et al., 1996; Phillis et al., 1996). The observations are suggestive of a functional link between EWG and DLC during *Drosophila* development.

These observations prompted us to determine whether EWG was functionally related to NRF-1 and whether DLC and EWG can interact. Since no *Drosophila* target genes for EWG have been identified, gel mobility shift assays were performed to determine whether EWG could bind an authentic NRF-1 recognition site. As shown in Fig. 8, nuclear extracts from cells

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**Fig. 6.** Subcellular distribution of DLC and NRF-1 determined by confocal laser scanning fluorescence microscopy. Balb/3T3 cells were processed for immunofluorescence microscopy using rabbit anti-DLC serum #76 (A,D,G) and monoclonal anti-HA antibodies (B,E,H). Confocal images are shown and green (DLC) and red (NRF-1) images were merged in C, F and I to visualize colocalization. See text for details. Box side represents 92 μm, 61 μm and 46 μm in A-C, D-E and G-I, respectively.
expressing EWG formed a DNA/protein complex that was easily distinguished from that formed with NRF-1. As expected, the EWG/DNA complex displayed slower mobility than the NRF-1/DNA complex (Fig. 8, lanes 1 and 7). Both complexes were competitively displaced by an excess of unlabeled NRF-1 oligonucleotide (lanes 6 and 12) but not by a non-specific oligonucleotide (lanes 5 and 11). In addition, the EWG complex was eliminated by the inclusion of anti-EWG serum (lane 10) but not by pre-immune (lane 8) or anti-NRF-1 serum (lane 9). These results demonstrate that EWG has the same DNA binding specificity as NRF-1.

It was also of interest to determine whether EWG had the same transcriptional specificity as NRF-1. This was done by comparing the ability of EWG and NRF-1 to trans-activate the NRF-1-dependent cytochrome c promoter in cultured Drosophila cells. These cells were used for trans-activation because they lacked endogenous NRF-1 and EWG (data not shown). As shown in Table 1, EWG expressed from a Drosophila actin promoter activated the cytochrome c promoter to a level higher than that observed for NRF-1 expressed from the same promoter. This result establishes that EWG, like NRF-1, can function as a transcriptional activator on an NRF-1-dependent promoter.

Given the structural and functional conservation between NRF-1 and EWG, it was possible that their ability to interact with DLC would also be conserved. To determine whether an EWG/DLC interaction can occur in vitro, EWG, NRF-1 and NRF-1/42-503 were radiolabeled by in vitro translation. Each protein was mixed with DLC and its ability to be co-immunoprecipitated with anti-DLC serum was assayed. The results show that both NRF-1 (Fig. 9A, lane 2) and EWG (lane 8) were present in immunoprecipitates under conditions where NRF-1/42-503 was not (lane 5). None of the proteins were precipitated by pre-immune serum (lanes 3, 6, and 9). Immunoprecipitations were also carried out using extracts from transfected cells expressing combinations of the various proteins followed by detection of DLC by immunoblotting as shown in Fig. 7. The results show that both NRF-1 (Fig. 9A, lane 2) and EWG (lane 8) were present in immunoprecipitates under conditions where NRF-1/42-503 was not (lane 5). None of the proteins were precipitated by pre-immune serum (lanes 3, 6, and 9). Immunoprecipitations were also carried out using extracts from transfected cells expressing combinations of the various proteins followed by detection of DLC by immunoblotting as shown in Fig. 7. The results show that both NRF-1 (Fig. 9A, lane 2) and EWG (lane 8) were present in immunoprecipitates under conditions where NRF-1/42-503 was not (lane 5). None of the proteins were precipitated by pre-immune serum (lanes 3, 6, and 9).

**DISCUSSION**

Nuclear transcription factors engage in a variety of protein-protein interactions in carrying out their biological functions. Here, we report an unexpected interaction between NRF-1 and DLC that was initially detected by yeast two-hybrid screening. DLC was isolated 14 times from two different mouse libraries using as bait a derivative of NRF-1 that was lacking its trans-activation domain. Moreover, two different mouse DLC isoforms were found. The mouse-a clones had sequences identical to DLC proteins isolated from human and rat. The mouse-b isoform represents a previously unknown DLC isoform and differs from the other mammalian DLCs at six amino acid positions. Interestingly, four of these six amino acids are shared with Drosophila DLC suggesting that the protein arose from a gene duplication that preceded the mammalian radiation. Mouse-b clones were all obtained from hybrid screening. DLC was isolated 14 times from two different mouse libraries using as bait a derivative of NRF-1 that was lacking its trans-activation domain. Moreover, two different mouse DLC isoforms were found. The mouse-a clones had sequences identical to DLC proteins isolated from human and rat. The mouse-b isoform represents a previously unknown DLC isoform and differs from the other mammalian DLCs at six amino acid positions. Interestingly, four of these six amino acids are shared with Drosophila DLC suggesting that the protein arose from a gene duplication that preceded the mammalian radiation. Mouse-b clones were all obtained from

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<td>pGL3RC4-326 pPac5c</td>
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*Construction of plasmids is described in Materials and Methods. In pGL3RC4-326, the expression of luciferase is directed by the proximal rat cytochrome c promoter. In pPac5c, expression is directed by Drosophila melanogaster actin 5c promoter. Activator plasmids contain either no coding region or the coding region for EWG or human NRF-1.

**Table 1. Trans-activation of the cytochrome c promoter by NRF-1 and EWG**

**Fig. 7. Co-immunoprecipitation of DLC and NRF-1. (A) Recombinant NRF-1 and DLC were mixed in PBS, 1%NP-40. Reaction mixtures were subjected to immunoprecipitation with anti-NRF-1 serum or anti-NRF-2β1 serum as a negative control as indicated above each lane. Immunoprecipitates were electrophoresed on a 15% polyacrylamide SDS-PAGE gel and DLC detected by immunoblotting using anti-DLC serum. (B) COS cells were transfected with pSG5DLC and pSG5NRF-1 as indicated. Whole cell lysates were prepared and subjected to immunoprecipitation with anti-NRF-1 serum or anti-NRF-2β1 serum as a negative control as indicated above each lane. Immunoprecipitates were electrophoresed on a 15% polyacrylamide SDS-PAGE gel and DLC detected by immunoblotting using anti-DLC serum. The positions of molecular mass standards are shown at the left in both panels.**

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†Fold activation is expressed as the mean of three experiments. The luciferase relative light units (RLU’s) of the pPac5c-activated pGL3RC4-326 is designated as ‘1’.
the mouse testis library raising the possibility that this isoform is testis specific.

Because two-hybrid screens can yield false positives, several independent criteria were applied to establish the authenticity of the NRF-1/DLC interaction. All of the initial positives were subcloned and retested for their ability to grow on –His medium and assayed for β-galactosidase activity. The specificity of the interaction was also tested against several unrelated ‘bait’ constructs as negative controls. The interaction was confirmed using full-length recombinant DLC and NRF-1 in an in vitro crosslinking reaction to establish that the full-length native proteins could interact. Both the two-hybrid and the chemical crosslinking assays identified the amino-terminal domain in NRF-1 as the site of DLC binding. This confirmed that the interaction between native proteins in vitro is the same as the interaction between the fusion proteins in the two-hybrid assay. NRF-1 and DLC also co-immunoprecipitated from cell extracts expressing both proteins as well as from in vitro mixtures of the two proteins. This demonstrates that the proteins can interact specifically in the presence of the full complement of potential competitors that are present in mammalian cells. Finally, it should be noted that DLC is not listed among the false positives in a database of two-hybrid artifacts (Hengen, 1997) (http://www.fccc.edu/research/labs/golemis/Table1.html) indicating that DLC does not interact promiscuously with a large number of unrelated proteins. The results are consistent with the conclusion that NRF-1 and DLC engage in a highly specific interaction both in vitro and in vivo.

The glutaraldehyde crosslinking assay revealed two distinct crosslinked species. One corresponded in size to the DLC dimer of approximately 19 kDa while a second, larger species migrated at 27 kDa, close to that expected for a trimer. DLC has been shown to dimerize in solution through an amphiphilic helical domain (Benashski et al., 1997). The dimerization is thought to occur through parallel interactions between dimerization helices. Crosslinked species of similar sizes to those observed here were also observed using DLC derived from myosin but the nature of the larger species was not investigated (Benashski et al., 1997).

A comparison between the sequences of the DLC interacting domains from neuronal nitric oxide synthase (Jaffrey and Snyder, 1996) and NRF-1 did not reveal significant sequence similarities. However, secondary structure predictions using a standard algorithm (Eisenberg et al., 1984) did reveal amphipathic α-helical regions within the NRF-1 amino-terminal domain that could serve as the sites of DLC interaction. The crosslinked NRF-1 dimer increases by approximately 20 kDa upon crosslinking to DLC (data not shown), a finding consistent with the conclusion that two DLC monomers or a DLC dimer interact with the N-termini of the NRF-1 dimer. However, in contrast to neuronal nitric oxide synthase, where the interaction with DLC was found to dissociate the catalytically active homodimer (Jaffrey and Snyder, 1996), we did not observe any change in NRF-1 dimerization upon addition of DLC (data not shown). Although the binding of DLC to neuronal nitric oxide synthase has recently been confirmed by a second laboratory, no dissociation of the dimer or inhibition of enzyme activity was observed (Rodriguez-Crespo et al., 1998). Thus, it seems unlikely that a general function for DLC is to regulate the monomer/dimer equilibrium of homodimeric proteins.

DLC was originally identified on the basis of its interaction with dynnein, a protein associated with the cytosolic microtubule network (King et al., 1996). NRF-1, on the other hand, was clearly localized to the cell nucleus (Gugneja et al., 1996). This raises the question of how DLC can interact with a nuclear transcription factor such as NRF-1. Recent findings demonstrated that, in mammalian brain, a substantial fraction of DLC was not associated with microtubules raising the possibility that DLC has other cellular functions (King et al., 1996). Moreover, a recent study has also shown that DLC is localized to both the nucleus and cytoplasm in mammalian cells (Crepieux et al., 1997). In this case, DLC was present in both nuclear and cytoplasmic extracts following subcellular fractionation of two different mammalian cell lines. This was confirmed by

![Fig. 8. Specific binding of EWG and NRF-1 to the same NRF-1 recognition site. COS cells were transfected with either pSG5NRF-1 or pSG5EWG and nuclear extracts were prepared for use in a gel electromobility shift assay. Lanes 1-6 show the shifted complexes formed using 1 μg of extract from NRF-1-expressing cells. Lanes 7-12 show the shifted complexes formed using 5 μg of extract from EWG-expressing cells. The positions of DNA/protein complexes formed with NRF-1 or EWG are indicated at the left. The NRF-1 complex present in EWG-expressing cells results from the presence of endogenous NRF-1. Complexes were formed using a 32P-labeled oligonucleotide containing the rat cytochrome c NRF-1 binding site (RC4-172/-147). The specific competitor was the same unlabeled oligonucleotide and the non-specific competitor was an unlabeled Serum Response Element (SRE) oligonucleotide (Lee et al., 1991) both added at 100-fold molar excess. Pre-immune (1 μl), anti-NRF-1 (1 μl) and anti-EWG (5 μl) sera were present in binding reactions as indicated above each lane.](http://www.fccc.edu/research/labs/golemis/Table1.html)
immunofluorescence staining of both nucleus and cytoplasm using an anti-DLC antibody. Here, we also demonstrate the presence of DLC in both the nucleus and cytoplasm by immunofluorescent staining. The fact that a nearly identical staining pattern was observed with antibodies directed against mouse and Chlamydomonas proteins argues that the nuclear localization is not an artifact of the antibody preparation. The same results were also obtained using methanol fixation (not shown). Moreover, the significant degree of nuclear co-localization of NRF-1 and DLC revealed by the yellow color in the fluorescent overlay observed using confocal laser scanning microscopy (Fig. 6C,F,I), is consistent with a diffuse nuclear localization for DLC and NRF-1. Thus, a fraction of the cellular DLC resides in the nucleus where it can come into contact with nuclear proteins such as NRF-1.

A major unanswered question is what is the functional significance of the NRF-1/DLC interaction? It should be noted that, even in the case of dynein, no function has been assigned to DLC and it is not known whether DLC is required for dynein motor activity. The recently described interactions of DLC with IkBα (Crepieux et al., 1997) and Bim (Puthalakath et al., 1999) point to a role for DLC in the intracellular transport of important regulatory proteins from cytoskeletal elements to the nucleus or mitochondria, respectively. The functional significance of the NRF-1/DLC interaction is supported by work with the NRF-1 related protein, EWG. Although EWG target genes have not yet been identified, we show that DNA binding and transcriptional specificities are conserved between EWG and NRF-1. This suggests that the two proteins share a high degree of functional as well as structural conservation. In addition, EWG was found to interact with DLC in a manner similar to that observed for NRF-1. In light of the similarities between NRF-1 and EWG, the conservation of the DLC interaction between insects and mammals lends credence to the argument that the interaction between these molecules is physiologically significant.

A potential genetic link between DLC and EWG, comes from work in Drosophila. A partial loss-of-function mutation in the DLC locus led to the disruption of sensory axon projections in Drosophila embryos whereas total loss-of-function mutation resulted in apoptotic cell death and embryonic lethality (Dick et al., 1996; Phillis et al., 1996). Interestingly, the DLC mutant phenotypes are remarkably similar to those reported for EWG. Partial loss-of-function mutations in EWG resulted in breaks in the central nervous system commissures and longitudinal tracts leading to aberrant intersegmental axonal projection pathways in the embryo (Desimone and White, 1993). Total loss-of-function mutations in EWG, like their DLC counterparts, were embryonic lethal. It is of interest in this context, that the NRF-1 gene in zebra fish is expressed in the eye and central nervous system of developing embryos. Its disruption gave a larval-lethal phenotype that was accompanied by defects in the development of the central nervous system (Becker et al., 1998).

In keeping with the similarities between DLC and EWG mutant phenotypes, the expression of DLC and EWG mRNAs was also co-localized during Drosophila development. In situ
hybridization using DLC cDNA localized DLC expression strongly to the central nervous system and the neurons of the chordotonal organs in the Drosophila embryo (Dick et al., 1996; Phillis et al., 1996). This pattern was identical to that obtained by in situ hybridization with EWG cDNA (Desimone and White, 1993). These data are consistent with the hypothesis that EWG and DLC work together in the development of the Drosophila nervous system.

Finally, it is tempting to speculate that the nuclear DLC functions as a component of an intranuclear transport system. Recent evidence points to the existence of structures within the nucleus, termed interchromatin granules, that may serve as sites for the storage or assembly of protein components involved in gene expression (Spector, 1996). Transcription factors may be assembled within nuclear structures and distributed to sites of active gene expression. Interactions with nuclear DLC may function in the dynamic distribution of proteins involved in gene expression to their sites of action within the nucleus.

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