Association of the TLX-2 Homeodomain and 14-3-3 Signaling Proteins*

(Received for publication, February 2, 1998, and in revised form, June 18, 1998)

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Homeodomain proteins play important roles in various developmental processes, and their functions are modulated by polypeptide cofactors. Here we report that both in vitro and in vivo, 14-3-3η is associated with the TLX-2 homeodomain transcription factor that is required for mouse embryogenesis. Expression of 14-3-3η shifts the predominant localization of TLX-2 in COS cells from the cytoplasm to the nucleus. TLX-2 and 14-3-3η are expressed in the developing peripheral nervous system with spatially and temporally overlapping patterns, and they are also coexpressed in PC12 cells. Increased expression of either gene by transfection considerably inhibited nerve growth factor-induced neurite outgrowth of PC12 cells, and cotransfection of both genes led to a synergistic effect of suppression. These findings define 14-3-3η as a functional modulator of the TLX-2 homeodomain transcription factor and suggest that the in vivo function of TLX-2 in neural differentiation is likely regulated by signaling mediated by 14-3-3η.

Homeobox genes encode an evolutionarily conserved superfamily of transcription factors that play vital roles in various aspects of development (1). A number of cofactors, including homeoproteins (2–4), specific transcription factors (5–7), general transcription factors (8–10), and other types of proteins (11, 12), have been identified to interact with homeodomain proteins. These protein/protein interactions modulate specific functions of homeodomain proteins, including their DNA binding affinities and specificities (3), transcription regulatory activities (13), and specific biological functions (11, 12).

14-3-3 proteins are highly related dimeric factors found in eukaryotic organisms, including yeast, Drosophila, plants, and mammals (14). Members of the 14-3-3 family are involved in regulation of the enzymatic activities of tyrosine and tryptophan hydroxylases and protein kinase C, exocytosis, and the cell cycle (14). Several important signaling proteins such as Raf-1, Bcr, phosphatidylinositol 3-kinase, and polyoma middle T antigen have been found to interact with 14-3-3 (15–23). A suggested role for 14-3-3 proteins is to function as scaffolds or adaptors to mediate interactions between different signaling proteins (24–26). It has been shown that 14-3-3 proteins can activate Raf-1 (15, 27) and that they are required for specific Ras/mitogen-activated protein kinase signaling pathways in yeast and Drosophila (28–31).

The TLX-2 homeobox gene belongs to the HOX11 family, in which three members, HOX11/Tlx-1, Tlx-2, and Tlx-3, have been identified (32, 33). The ectopic activation of HOX11/Tlx-1 in T cells by chromosomal translocations results in malignant transformation (34–37). Gene targeting experiments have shown that Tlx-1 is required for the formation of mouse spleen (38, 39), whereas Tlx-2 is required for mouse gastrulation and mesoderm formation.1 During mouse embryogenesis, Tlx-2 is expressed in the primitive streak during gastrulation, in the neural ectoderm and the neural fold during neurulation, and later in the developing nervous system.1 To obtain insight into the regulation of TLX-2 function, we searched for proteins that associate with TLX-2 in vitro. This paper describes an in vitro and in vivo interaction between TLX-2 and the mouse 14-3-3η protein. The biological relevance of this interaction has been established at multiple levels: 14-3-3η enhances the nuclear localization of TLX-2; Tlx-2 and 14-3-3η are coexpressed in the developing PNS2 and in PC12 cells; and Tlx-2 and 14-3-3η cooperatively suppress neurite outgrowth of PC12 cells. Our findings provide the first evidence that a 14-3-3 protein can act as a cofactor for homeodomain transcription factors and suggest that the function of the TLX-2 homeodomain protein is modulated by specific signaling mediated by 14-3-3 proteins.

MATERIALS AND METHODS

Plasmid Constructs—pEXlox(+)Tlx-2 is a Tlx-2 cDNA plasmid derived from AElox-Tlx-2 after Cre-mediated excision (Novagen). The Tlx-2 cDNA fragment was flanked by EcoRI and HindIII sites, with the 5′-end closed to EcoRI. For pGEM7-Tlx-2, the EcoRI-HindIII fragment (the HindIII end was bluntly cloned by the Klenow fragment) from pEXlox(+)Tlx-2 was cloned into the EcoRI/SmaI sites of pGEM7. For pBSK5(−)Tlx-2/SacI, the 284-bp pair SacII fragment from the Tlx-2 genomic clone was cloned into the SacII site of pBSK5(−)SacI, the 284-bp pair SacII fragment from the Tlx-2 genomic clone was cloned into the SacII site of pBSK5(−)SacI, the 284-bp pair SacII fragment from the Tlx-2 genomic clone was cloned into the SacII site of pBSK5(−)SacI, the 284-bp pair SacII fragment from the Tlx-2 genomic clone was cloned into the SacII site of pBSK5(−)SacI, the 284-bp pair SacII fragment from the Tlx-2 genomic clone was cloned into the SacII site of pBSK5(−)SacI. For pGST2TK-Tlx-2, the Nov-1/HindIII Tlx-2 fragment was released from pGEM7-Tlx-2, bluntly by the Klenow fragment, and cloned into the SmaI site of pGEX2TK in frame with GST. For pGST2TK-Tlx-2, the Tlx-2 fragment in pGST2TK-Tlx-2 was flanked by EcoRI and HindIII sites, with the 5′-end closed to EcoRI. For pGST2TK-Tlx-2, the Tlx-2 fragment in pGST2TK-Tlx-2 was flanked by EcoRI and HindIII sites, with the 5′-end closed to EcoRI. For pGST2TK-Tlx-2, the Tlx-2 fragment in pGST2TK-Tlx-2 was flanked by EcoRI and HindIII sites, with the 5′-end closed to EcoRI.

1 S. J. Tang, T.-C. Suen, R. R. McInnes, and M. Buchwald, unpublished results.

2 The abbreviations used are: PNS, peripheral nervous system; GST, glutathione S-transferase; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; NGF, nerve growth factor; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.
fragment from pGEM7-Tlx-2 was cloned into the ClaI/BamHI sites of pFLAG-CMV2 (ClaI and BamHI were blunted). pEXlox(+)-14-3-3 was a mouse 14-3-3 cDNA plasmid derived from AElox-14-3-3 after Cre-mediated excision. For pGEM7-14-3-3, the EcoRI-NsiI fragment of 14-3-3 from pEXlox(+)-14-3-3 was cloned into the EcoRI/NsiI sites of pGEM7. For pcDNA-14-3-3, the EcoRI-NsiI fragment of 14-3-3 from pEXlox(+)-14-3-3 was cloned into the EcoRI/NsiI sites of pcDNA-1. For pGST2TK-14-3-3, the pEXlox(+)-14-3-3 EcoRI-NruI fragment was blunted with the Klenow fragment, followed by self-ligation. The EcoRI site was restored by the self-ligation. The EcoRI-NruI fragment (EcoRI was blunted by the Klenow fragment) was cloned into the BamHI/PstI sites (BamHI was blunted by the Klenow fragment) of pBTM116 to construct pBTM116–14-3-3. For co-immunoprecipitations, COS-7 cells grown on a 100-mm plate in Dulbecco's modified Eagle's medium were transfected with expression vectors encoding FLAG-14-3-3 and TLX-2. The supernatant was precleared by incubation with 20 μl of agarose beads for 2 hours at 4°C. The beads were washed four times with 1 ml of 0.1% Triton X-100 in PBS. The protein bound to the beads was eluted with 1× Laemmli loading buffer and separated on 4–20% SDS-polyacrylamide gradient gels (Novex) in Tris/glycine buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS). After drying, the gel was exposed to x-ray film overnight at room temperature. For co-immunoprecipitations, COS-7 cells grown on a 100-mm plate in Dulbecco's modified Eagle's medium were transfected with expression vectors encoding 14-3-3 and TLX-2 using calcium phosphate and were harvested 30 h later. Cells were washed once in PBS, resuspended in 500 μl of TNM buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM MgCl2, and 0.1% Nonidet P-40) containing complete proteinase inhibitor mixture (Boehringer Mannheim), and sonicated 3 × 10 s on ice. The cell lysate was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was precleared by incubation with 20 μl of protein A-conjugated agarose beads (50% slurry) for 30 min at 4°C. Co-immunoprecipitation was performed by incubating the supernatant with 1 μg of anti.FLAG M2 antibody (Eastman Kodak Co.) conjugated to agarose beads for 2 hours at 4°C. The beads were washed four times with 1 ml of TNM buffer, resuspended in 40 μl of Laemmli loading buffer, and boiled for 2 min. For Western blot analysis, 10 μl of the co-immunoprecipitated product was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes overnight (4°C, 30 V) in transfer buffer (20 mM Tris-HCl (pH 7.5), 150 mM glycine, and 20% methanol). After transfer, the membrane was incubated in PBS containing 0.2% Triton X-100 at room temperature with gentle shaking. The membranes were washed three times for 5 min in PBS containing 0.2% Triton X-100 at room temperature with gentle shaking. The membranes were air-dried and exposed to x-ray film overnight at –80°C.

**In Vitro Protein Binding Assay, Co-immunoprecipitation, and Western Blot Analysis—**In vitro binding assays, 1 pmol of GST or GST fusion proteins bound to glutathione-Sepharose 4B beads was incubated with in vitro translated TLX-2 or 14-3-3 labeled with [35S]methionine (Amersham Pharmacia Biotech) at 4°C for 2 h. The beads were washed three times with 1 ml of 0.1% Triton X-100 in PBS. The protein bound to the beads was eluted with 1× Laemmli loading buffer and separated on 4–20% SDS-polyacrylamide gradient gels (Novex) in Tris/glycine buffer (25 mM Tris, 250 mM glycine, and 0.1% SDS). After drying, the gel was exposed to x-ray film overnight at room temperature. For co-immunoprecipitations, COS-7 cells grown on a 100-mm plate in Dulbecco's modified Eagle's medium were transfected with expression vectors encoding 14-3-3 and TLX-2 using calcium phosphate and were harvested 30 h later. Cells were washed once in PBS, resuspended in 500 μl of TNM buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM MgCl2, and 0.1% Nonidet P-40) containing complete proteinase inhibitor mixture (Boehringer Mannheim), and sonicated 3 × 10 s on ice. The cell lysate was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was precleared by incubation with 20 μl of protein A-conjugated agarose beads (50% slurry) for 30 min at 4°C. Co-immunoprecipitation was performed by incubating the supernatant with 1 μg of anti-FLAG M2 antibody (Eastman Kodak Co.) conjugated to agarose beads for 2 hours at 4°C. The beads were washed four times with 1 ml of TNM buffer, resuspended in 40 μl of Laemmli loading buffer, and boiled for 2 min. For Western blot analysis, 10 μl of the co-immunoprecipitated product was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes overnight (4°C, 30 V) in transfer buffer (25 mM Tris-HCl (pH 8.3), 150 mM glycine, and 20% methanol). After transfer, the membrane was incubated in PBS, blocked in PBS containing 0.1% Tween-20 and 5% nonfat milk (Bio-Rad) for 1 h at room temperature, washed three times (1 × 15 min, 2 × 5 min) with wash buffer (0.1% Tween-20 in PBS), and then incubated with the anti-TLX-2 polyclonal antibody for 1 h at room temperature. After three washes (1 × 15 min, 2 × 5 min), the membrane was incubated with peroxidase-labeled anti-rabbit antibody (Amersham Pharmacia Biotech) for 30 min at room temperature, followed by five washes (1 × 15 min, 4 × 5 min). Detection was performed with enhanced chemiluminescence Western blotting detection reagents (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions and verified by SDS-polyacrylamide gel electrophoresis. Fifty ng of the purified fusion protein was phosphorylated by muscle kinase in 35 μl of reaction buffer (20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 100 mM NaCl, 12 mM MgCl2, 5 μl of [γ-32P]ATP (6000 Ci/mmol; DuPont NEG-0022) for 30 min at 30°C. The labeling reaction was passed through a Sephadex G-25 column (equilibrated in PBS containing 0.1% bovine serum albumin) to separate the fusion protein from the free isotope. The specific activity of the probe was usually at the range of 5–8 × 106 cpm/μg. A mouse E10.0 embryonic cDNA expression library (Novagen) was plated according to the manufacturer's instructions at a density of 4–5 × 104 plaques/150-mm agarose plate. After 3 h, nitrocellulose membranes (Amersham Pharmacia Biotech) presoaked in 1× isopropyl-β-D-thiogalactopyranoside were placed on the plates, and the incubation was continued overnight. The membranes were lifted, rinsed in PBS with 0.05% Triton X-100, and blocked in prehybridization solution (20 mM HEPES (pH 7.5), 5 mM MgCl2, and 1 mM KCl) containing 5% blocking reagent (Bio-Rad) for 2 h at 4°C. After prehybridization, the membranes were transferred into hybridization solution (pre-hybridization solution plus probe at a concentration of ~5 × 106 cpm/ml) and incubated at 4°C overnight with gentle shaking. The membranes were then washed three times for 5 min in PBS containing 0.2% Triton X-100 at room temperature with gentle shaking. The membranes were air-dried and exposed to x-ray film overnight at –80°C.

**Screening of the cDNA Expression Library—**This method was modified from techniques described before (40, 41). The GST-TLX-2/68 copy of 14-3-3 was cloned into the BamHI/PstI sites (BamHI was blunted by the Klenow fragment) of pBTM116 to construct pBTM116–14-3-3. The Smal-BglII fragment from pBTM116–14-3-3 was released, blunted by the Klenow fragment, and cloned into the Smal site of pGEM7. The 5′-3′ linker fragment from pGEM7–14-3-3 was cloned into the HindIII/BglII sites (HindIII was blunted by the Klenow fragment) of the pFLAG-CMV2 vector.

**Fig. 1. Mouse 14-3-3 cDNA.** Shown are the nucleotide and deduced amino acid sequences of the mouse 14-3-3 cDNA.
The TLX-2 rabbit antiserum was raised against the synthetic peptide AEDNKVASVSGL, corresponding to the C terminus of TLX-2 protein (produced by the National Centers of Excellence, Vancouver, Canada). The specificity of this antiserum was tested with GST-TLX-2 and in vitro translated TLX-2 protein (data not shown).

**Indirect Immunofluorescence Staining**—COS-7 cells cultured in Permanox chamber slides (Nunc) were transiently transfected with either FLAG-TLX-2 or FLAG-14-3-3h alone or with both pcDNA-14-3-3h and pcDNA-Tlx-2. Cells were washed once with PBS and fixed for 10 min with 4% paraformaldehyde in PBS, followed by three washes with PBS. Fixed cells were then permeabilized with cold (−20 °C) methanol for 2 min, washed four times with PBS, blocked in 10% goat serum for 1 h at room temperature, and then incubated overnight at 4 °C in 10% goat serum containing 20 μg/ml anti-FLAG M2 monoclonal antibody. Cells were washed four times with PBS and then incubated for 2 h at room temperature with FITC-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, Inc.) in 10% goat serum, followed by three subsequent washes. For nuclear staining, cells were incubated in 4,6-diamidino-2-phenylindole (1 mg/ml in PBS) for 5 min and washed three times in PBS.

**Whole Mount RNA in Situ Hybridization**—Whole mount in situ hybridization was performed as described (42). For generating the Tlx-2 antisense riboprobe, pGEM7-Tlx-2/SacII A was linearized with NotI and transcribed with T7 polymerase. For generating the 14-3-3h antisense riboprobe, pEXlox(1)-14-3-3h was linearized with BglII and transcribed with Sp6 polymerase.
SDS at room temperature, followed by two washes in 0.2× SSC and 0.2% SDS at 68 °C.

**RESULTS**

Interaction Cloning of the Mouse 14-3-3 Gene—The Tlx-2 gene is specifically expressed in the developing mouse PNS at day 10.0 of mouse gestation.1 To isolate proteins that interact with and regulate TLX-2 during PNS ontogenesis, a GST-TLX-2 fusion protein containing the C-terminal 68 residues of TLX-2 was32P-labeled with heart muscle kinase and used to screen a cDNA expression library derived from mouse embryos 10.0 days postcoitus (Novagen) (40, 41). A positive clone was isolated after four rounds of purification from 2.5×10^5 plaques screened. Sequence analysis revealed that this cDNA clone contained a fragment of 1668 base pairs (Fig. 1), which was similar to the size of its mRNA as determined by Northern blot analysis (see Fig. 5A). A GenBank™ search using the deduced amino acid sequence of the open reading frame revealed that the cloned gene was the ortholog of rat 14-3-3 (43).

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Vivo—The results of interaction cloning described above suggested physical interaction between the C terminus of TLX-2 and the 14-3-3\(h\) protein. To confirm the interaction between the full-length proteins, in vitro assays were performed using GST-TLX-2 and GST-14-3-3\(h\) fusion proteins and in vitro translated TLX-2 and 14-3-3\(h\) (Fig. 2A). The results showed that \(^{35}\)S-labeled TLX-2 and 14-3-3\(h\) bound to GST-14-3-3\(h\) and GST-TLX-2, respectively, but not to GST (Fig. 2B). Consistent with previous observations on the dimerization of 14-3-3 proteins (44), an intermolecular interaction was also detected for the 14-3-3\(h\) protein (Fig. 2B).

To determine if the association between TLX-2 and 14-3-3\(h\) proteins occurs in mammalian cells, co-immunoprecipitation was performed on extracts from COS-7 cells transiently co-transfected with expression vectors encoding FLAG epitope-tagged 14-3-3\(h\) and wild-type TLX-2. Precipitation was performed using the antibody against the FLAG epitope, followed by Western blot analysis with antibodies against TLX-2. As shown in Fig. 2C, the TLX-2 protein was coprecipitated with 14-3-3\(h\), indicating that these two proteins associate in mammalian cells.

Enhancement of TLX-2 Nuclear Localization by 14-3-3\(h\)—To identify the subcellular compartment in which TLX-2 and 14-3-3\(h\) interact, we first determined the cellular localization of TLX-2 and 14-3-3\(h\) in COS-7 cells transfected with an expression vector encoding FLAG epitope-tagged TLX-2 (FLAG-TLX-2). After staining with anti-FLAG primary and FITC-conjugated secondary antibodies, two patterns of fluorescent signals were observed in the cell population transfected with FLAG-TLX-2: exclusive nuclear staining was seen in 36% of the FITC-positive cells, whereas the remaining 64% cells had signals in both the cytoplasm and nucleus (Fig. 3, A, panels a and c; and C). When COS-7 cells were transfected with an expression vector coding for FLAG-14-3-3\(h\), the fluorescent signal was observed primarily in the cytoplasm and on the cell membrane (Fig. 3B, panels e and g). These results demonstrate a co-localization of TLX-2 and 14-3-3\(h\) in the cytoplasm. To determine if the interaction of 14-3-3\(h\) and TLX-2 affected the cellular localization of TLX-2, we examined the pattern of fluorescent signals in COS-7 cells cotransfected with FLAG-TLX-2 and 14-3-3\(h\). When mouse Tlx-2 and 14-3-3\(h\) are expressed in this cell line (see below). After cotransfection, we observed that the majority of the FLAG-TLX-2-positive COS cells (86%) had exclusively nuclear signals with stronger signals in the perinuclear regions, whereas the remaining fraction (14%) had signals in both the cytoplasm and nucleus (Fig. 3, A, panels b and d; and C). The shift of the localization pattern of TLX-2 proteins in the presence of 14-3-3\(h\) demonstrates that 14-3-3\(h\) significantly enhances the nuclear localization of TLX-2 in COS cells (0.005 < \(p\) < 0.01). However, coexpression of TLX-2 did not alter the cellular distribution of 14-3-3\(h\) (Fig. 3B, panels f and h).

**Overlapping Expression Patterns of 14-3-3\(h\) and Tlx-2 in the**
Developing PNS—To assess the biological significance of the interaction between TLX-2 and 14-3-3 during development, whole mount in situ hybridization analysis was performed to compare the expression patterns of these two genes during embryogenesis. As shown in Fig. 4, Tlx-2 and 14-3-3 were detected extensively overlapping expression domains in the developing PNS, including both sensory and autonomic systems (Fig. 4; data not shown). Compared with that of Tlx-2, the expression pattern of 14-3-3 was broader. In addition to the developing PNS, 14-3-3 expression was also detected in other tissues and organs, including branchial arches and limb buds (Fig. 4). These results demonstrate the coexpression of Tlx-2 and 14-3-3 in the developing PNS and suggest a functional interaction between these two proteins during PNS formation.

Coexpression of Tlx-2 and 14-3-3 in PC12 Cells—PC12 cells are derived from transformed adrenal chromaffin cells that originate, as do most components of the PNS, from the neural crest. These cells can differentiate into sympathetic-like neurons after NGF induction (45). PC12 cells were therefore used to assess the functional interaction between Tlx-2 and 14-3-3. We first examined if these genes are coexpressed in PC12 cells. Results of the Northern blot analysis showed that both genes are expressed in this cell line before and after NGF induction (Fig. 5A).

Synergistic Suppression of the Neurite Outgrowth of PC12 Cells by TLX-2 and 14-3-3—Previous studies have demonstrated an inhibitory effect of Tlx-2 overexpression on NGF-induced neurite outgrowth of PC12 cells. We therefore examined the effect of 14-3-3 on this activity of Tlx-2 by cotransfection. PC12 cells were first transfected with expression vectors of Tlx-2 and 14-3-3 together with RSV-LacZ, as indicated in Table I. After 3 days of NGF induction, the transfected cells were identified by LacZ staining and scored according to the relative length of their neurites. Consistent with previous observations, transfection of Tlx-2 led to substantial suppression of the NGF-induced neurite outgrowth (Table I). Moreover, transfection of 14-3-3 alone also caused a substantial suppression of the NGF-induced neurite growth (Table I). More important, when both Tlx-2 and 14-3-3 were cotransfected into PC12 cells, a synergistic suppression of neurite outgrowth was observed (Fig. 5B). This result suggests a functional interaction between Tlx-2 and 14-3-3 proteins during neuronal differentiation of PC12 cells. The physiological relevance of this observation is supported by the coexpression of Tlx-2 and 14-3-3 in PC12 cells and in the developing PNS. Although single transfection of human HOX11/TLX-1 resulted in a moderate suppression of neurite outgrowth, we did not observe a synergistic effect when TLX-1 and 14-3-3 were cotransfected (Fig. 5C).

We also assessed the specific stage of neurite growth affected by TLX-2 and 14-3-3. In mock transfections, the percentage of transfected cells without processes was considerably lower after 6 days of induction than after 3 days (Table I), indicating that active neurite outgrowth was occurring in this group of transfected cells during this period. In contrast, the percentage of cells without processes in cells transfected with either Tlx-2 or 14-3-3 alone or cotransfected with both vectors did not decrease as the induction proceeded, suggesting that these transfected cells did not initiate neurite outgrowth. On the other hand, the percentage of cells with processes longer than one cell body in both mock and experimental transfections increased from days 3 to 6, whereas the percentage of cells with neurites shorter than one cell body decreased substantially. This indicates that the growth of existing processes continued in cells transfected with Tlx-2 and 14-3-3, although it is difficult to determine if the rate of growth of pre-existing neurites is the same as in mock-transfected cells (Table I). Together, these data suggest a role for both Tlx-2 and 14-3-3 in blocking the initiation of neurite outgrowth.
Relative length of neurites were as follows: 0, without neurites; <1, neurites shorter than one cell body; 1–2, neurites longer than one but shorter than two cell bodies; >2, neurites longer than two cell bodies. RSV-LacZ plasmid was included in each transfection, and the transfected cells were identified by LacZ staining. pcDNA-1 transfection was included as a control.

**TABLE I**

| Relative length of neurites | pcDNA-1 | Tlx-2 | 14–3-3°C | Tlx-2 + 14–3-3°C |
|-----------------------------|---------|-------|----------|------------------|
| 0                           | 36.8 ± 0.6  | 59.4 ± 1.8  | 59.6 ± 4.6  | 90.6 ± 1.8       |
| <1                          | 44.1 ± 0.1  | 30.3 ± 1.7  | 34.1 ± 3.2  | 8.5 ± 1.3        |
| 1–2                         | 14.9 ± 0.9  | 8.3 ± 0.2   | 5.0 ± 1.3   | 0.6 ± 0.6        |
| >2                          | 4.3 ± 0.4   | 2.1 ± 0.1   | 1.6 ± 0.4   | 0.4 ± 0.0        |
| Total cells                 | 465       | 437     | 438       | 504              |

### DISCUSSION

*In vitro* and *in vivo* interactions between the TLX-2 homeodomain and 14-3-3°C proteins have been demonstrated in this study. The biological significance of the interaction is supported by several observations: 14-3-3°C increases the nuclear localization of TLX-2; Tlx-2 and 14-3-3°C are coexpressed in the developing PNS and in PC12 cells; and TLX-2 and 14-3-3°C cooperatively suppress neurite outgrowth of PC12 cells induced by NGF. Recent studies have suggested that 14-3-3°C proteins can bind to the phosphoserine in an RSXpSXP or RX(Y/F)XpSXP motif in various signaling proteins (46, 47). However, such motifs are not present in the amino acid sequence of TLX-2, indicating that a different binding mechanism or motif mediates its interaction with 14-3-3°C. Notably, these motifs are also not found in the peptide of platelet glycoprotein Ibα that mediates its interaction with 14-3-3°C (48), and the two motifs (NRHpsLTP and RLpSTF) that are implicated in 14-3-3 binding of Cbf share little homology with the above consensus binding sites, but can cooperatively confer stable binding to 14-3-3 (49). Nonetheless, serine-rich regions are present in both the N- and C-terminal portions of TLX-2 (32). It will be interesting for further experiments to determine whether the interaction between TLX-2 and 14-3-3°C proteins is also phosphoserine-dependent.

Recent studies have identified a variety of cofactors for homeodomain transcription factors; many of them are also involved in transcription regulation (see the Introduction). Although modulation of the DNA binding affinity and specificity of homeoproteins by their cofactors has been strongly suggested, the functional significance of their interactions is often unclear in a biological context. Our findings of the association of 14-3-3°C with TLX-2 define a new class of cofactor for homeodomain transcription factors. Given the well-established role of its cellular distribution by signaling mediated by 14-3-3, it is likely that TLX-2 may interact with one or more of those kinases. Since phosphorylation and dephosphorylation serve as a major mechanism regulating the nuclear localization of transcription factors (53), the observed enhancement of TLX-2 nuclear localization by 14-3-3°C may be attributed to a 14-3-3-mediated interaction between TLX-2 and a signaling kinase that modulates the phosphorylation state of TLX-2. Taken together, our results suggest that one likely mechanism that controls the developmental function of TLX-2 is the regulation of its cellular distribution by signaling mediated by 14-3-3 proteins.

**Acknowledgments**—We are grateful to Dra. J. Lightfoot, C. C. Hui, and M. Crackower for critical reading of the manuscript.

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J. Biol. Chem. 1998, 273:25356-25363.
doi: 10.1074/jbc.273.39.25356

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