Molecular Analysis of the X11–mLin-2/CASK Complex in Brain

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A heterotrimetric complex containing Lin-10/X11α, Lin-2/CASK, and Lin-7 is evolutionarily conserved from worms to mammals. In Caenorhabditis elegans, it localizes Let-23, a receptor tyrosine kinase, to the basolateral side of vulval epithelium, a step crucial for proper vulva development. In mammals, the complex may also participate in receptor targeting in neurons. Accordingly, phosphotyrosine binding (PTB) and postsynaptic density-95/Discs large/Zona Ocludens-1 domains found in X11α and mLin-2/CASK bind to cell-surface proteins, including amyloid precursor protein, neurexins, and syndecans. In this paper, we have further analyzed the X11α–mLin-2/CASK association that is mediated by a novel protein–protein interaction. We show that the mLin-2/CASK calmodulin kinase II (CKII) domain directly binds to a 63 amino acids peptide located between the Munc-18-1 binding site and the PTB domain in X11α. Calmodulin association with mLin-2/CASK does not modify the X11α–mLin-2 interaction. A region containing the mLin-2/CASK guanylate kinase domain also interacts with X11α but with a lower affinity than the CKII domain. Immunostaining of X11α in the brain shows that the protein is expressed in areas shown previously to be positive for mLin-2/CASK staining. Together, our data demonstrate that the X11α–mLin-2 complex contacts many partners, creating a macrocomplex suitable for receptor targeting at the neuronal plasma membrane.

Key words: PDZ; PTB; X11; mLin-2/CASK; CaM kinase; receptor localization
several brainstem nuclei. Cell-surface proteins, such as neurexins, syndecans, and APP, interact with the X11α–mLin-2/CASK complex through PTB and PDZ domain interactions (Borg et al., 1996; Hata et al., 1996; Cohen et al., 1998; Hsu et al., 1998).

Additional binding of mLin-7, Munc-18-1–Syntacin, and calmodulin generates a neuronal multiprotein complex, which we predict will be involved in receptor localization.

**MATERIALS AND METHODS**

**Antibodies.** Anti-Myc 9E10 (Oncogene Research Products, Cambridge, MA) monoclonal antibody was used for immunoprecipitation and immunoblotting. Polyclonal anti-mLin-2/CASK and anti-X11 antibodies were described previously (Borg et al., 1998a). Anti-PSD-95 and anticalmodulin monoclonal antibodies were from Upstate Biotechnology (Lake Placid, NY). Anti-T7 monoclonal antibody was from Novagen (Madison, WI). Anti-Syntaxin monoclonal antibody was from Sigma (St. Louis, MO). Anti-Munc-18-1 and anti-mLin-2/CASK monoclonal antibodies were from Transduction Laboratories (Lexington, KY). Avidin–biotin blocking kit, biotinylated goat anti-rabbit IgG, and streptavidin biotinylated horseradish peroxidase complex were purchased from Vector Laboratories (Burlingame, CA).

**DNA constructs.** Full-length X11α, X11β, and X11γ DNA have been described elsewhere (Borg et al., 1998b). Human lin-2 cDNA synthesized from expressed sequence tags (identical to GenBank accession number AF035582) (Borg et al., 1998a) was used as a template to create different DNA constructs. The RK5-myc vector was used to express X11α and mLin-2/CASK fused to the myc epitope (Borg et al., 1996). All constructs were sequenced using Sequenase version 2.0 (Amersham, Cleveland, OH).

**Cell culture.** Human embryonic kidney 293 and A-172 cells were grown in DMEM (Life Technologies, Grand Island, NY) containing 100 U/ml penicillin and 0.1% streptomycin sulfate, supplemented with 10% fetal calf serum (FCS). The sections were coverslipped and placed inside a humidified box overnight at 55°C. After hybridization, the coverslips were removed, and the sections were washed twice in 2× SSC for 5 min each and then incubated for 1 hr in RNase (200 μg/ml in Tris buffer containing 0.5 μM NaCl, pH 8.0) at 37°C. The sections were washed in increasingly stringent solutions of 2×, 1×, and 0.5× SSC for 5 min each, followed by incubation for 1 hr in 0.1× SSC, 5°C. After rinsing, the sections were dehydrated through graded series of alcohol, air dried, and exposed to a Kodak XAR film (Eastman Kodak, Rochester, NY) for 5–7 d. Finally, the sections were dipped into photographic emulsion (Kodak NTB-2), exposed for 13–17 d, developed in Kodak D-19 developer (2 min), fixed (3 min), and counter-stained with cresyl violet. Sections pretreated for 1 hr in RNase (200 μg/ml) and then washed with the same protocol and sense riboprobe from the same plasmid insert were used as controls.

**Immunostaining of NT2 cells.** Differentiated NT2 cells were plated on acid-treated coverslips coated with poly-D-lysine (Sigma) and Matrigel (Collaborative Research, Bedford, MA). After fixation with PBS–4% paraformaldehyde, cells were washed with PBS–10 μM glycine and permeabilized with PBS–0.1% TX. After blocking for 1 hr in goat serum, coverslips were incubated with antibodies diluted in PBS–2% goat serum overnight. The sections were then washed twice in 2× SSC for 5 min each and then incubated for 1 hr in RNase (200 μg/ml in Tris buffer containing 0.5 μM NaCl, pH 8.0) at 37°C. The sections were washed in increasingly stringent solutions of 2×, 1×, and 0.5× SSC for 5 min each, followed by incubation for 1 hr in 0.1× SSC, 5°C. After rinsing, the sections were dehydrated through graded alcohols, air dried, and exposed to a Kodak XAR film (Eastman Kodak, Rochester, NY) for 5–7 d. Finally, the sections were dipped into photographic emulsion (Kodak NTB-2), exposed for 13–17 d, developed in Kodak D-19 developer (2 min), fixed (3 min), and counter-stained with cresyl violet. Sections pretreated for 1 hr in RNase (200 μg/ml) and then washed with the same protocol and sense riboprobe from the same plasmid insert were used as controls.

**Immunohistochemistry.** Five male Sprague Dawley rats from Charles River Laboratories (Wilmington, MA), weighing 250–325 gm, were used in this study. The rats were anesthetized with sodium pentobarbitual (50 mg/kg, i.p.) (Butler, Columbus, OH) and perfused transcardially with 0.1% TX in PBS, followed by incubation in a solution of 5% formaldehyde, 0.1% bovine serum albumin (BSA), and 0.3% Triton X-100 (TX) in PBS for 20 min. After rinsing with distilled water (DW), the sections were dehydrated through graded alcohols, air dried, and exposed to a Kodak XAR film (Eastman Kodak, Rochester, NY) for 5–7 d. Finally, the sections were dipped into photographic emulsion (Kodak NTB-2), exposed for 13–17 d, developed in Kodak D-19 developer (2 min), fixed (3 min), and counter-stained with cresyl violet. Sections pretreated for 1 hr in RNase (200 μg/ml) and then washed with the same protocol and sense riboprobe from the same plasmid insert were used as controls.

**Protein procedures.** Cells were washed twice with cold PBS and lysed in lysis buffer (50 mM HEPES, pH 7.5, 10% glycerol, 150 mM NaCl, 1% TX, 1.5 mM MgCl2, and 1 mM EGTA) supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. After centrifugation at 16,000 × g for 20 min, lysate protein content was normalized using the Bio-Rad (Hercules, CA) protein assay kit. Mouse brain proteins were extracted after a similar procedure. For immunoprecipitation, lysates were incubated with antibodies overnight at 4°C. Protein A–agarose was added, and immune complexes bound to beads were recovered after 1 hr, washed three times with buffer (containing 30 mM HEPES, pH 7.5, 10% glycerol, 150 mM NaCl, and 0.1% TX), boiled in 1× sample buffer, and separated by SDS-PAGE. Transfer and immunoblotting on nitrocellulose using HRP protein A or HRP anti-mouse antibody chemiluminescence method were performed as described previously (Borg et al., 1996). For overlay assays, the membrane was incubated 2 hr at room temperature with soluble His-tagged or GST fusion proteins at 1 μg/ml in TBS–5% BSA and 1 mM DTT. After rinsing with TBS–0.1% TX and TBS–1% buffers, the membrane was incubated with mouse monoclonal anti-His antibody, which was followed by 2 hr of incubation with TBS–5% BSA for 2 hr. The immuno complexes were revealed using HRP goat anti-mouse antibody or HRP protein-A chemiluminescence method. Cell transfection, GST production, and GST binding assays were performed as described previously (Borg et al., 1996).
respectively. Proteins comprise the PTB and the two PDZ domains of the protein, respectively.

proteins. A central PTB domain, followed at its C-terminal end by two GST X11 and the X11 protein contains a central PTB domain, followed at its C-terminal end by two GST X11 and the X11 protein, respectively. Proteins comprise the PTB and the two PDZ domains of the protein, respectively.

In contrast, we found no interaction between Munc-18-1 or Syntaxin (Okamoto and Sudhof, 1997). In mLin-2/CASK from mouse brain extracts (Fig. 1A), Proteins extracted from mouse brain were precipitated with GST, GST X11 (region 163–346), GST X11β (region 140–415), or GST X11γ (region 15–246) coupled to glutathione beads. These fusion proteins incorporate peptides from the N terminus of these X11 isoforms. After washing, proteins were separated on 10% SDS-PAGE and transferred to nitrocellulose. The membrane was probed with polyclonal anti-mLin-2/CASK and monoclonal anti-Munc-18-1, anti-Syntaxin, and anti-PSD-95 antibodies. One-tenth of the lysate used for precipitation was run as control (lysate). Detection was performed by chemiluminescence. B, Same as A, but with additional X11α fusion proteins. The GST X11α PTB and PDZ domains of the protein, respectively.

RESULTS

Mapping the X11α—mLin-2 binding sites

We have further delineated the site of interaction on the X11α N terminus for mLin-2/CASK (Fig. 1A). In X11α, the 163–346 amino acids region is responsible for the coimmunoprecipitation between the two proteins (Borg et al., 1998a) and precipitates mLin-2/CASK from mouse brain extracts (Fig. 1B). Analogous regions in X11β and X11γ do not interact with mLin-2/CASK. As shown previously, X11α and X11β N termini bind to the complex of Munc-18-1 and Syntaxin (Okamoto and Sudhof, 1997). In contrast, we found no interaction between Munc-18-1 or Syntaxin and the X11γ N terminus. PSD-95 did not bind to X11 fusion proteins (Fig. 1B). We have generated GST fusion proteins representing smaller peptides of X11α and examined the binding to mLin-2/CASK by GST precipitation (Fig. 1C; Table 1). We demonstrate that a region encompassing residues 373–436 in X11α is sufficient to bind mLin-2/CASK. No binding was detected with the X11α PTB and PDZ domains (Fig. 1C). The binding site was further subdivided in shorter peptides, but none of them could bind to mLin-2/CASK, suggesting that the X11α region 373–436 represents the minimal site of interaction (Table 1). As expected, no homologous regions are found in X11β and X11γ. Others have found that the region 226–314 in X11α is sufficient to bind Munc-18-1 (Okamoto and Sudhof, 1997). Accordingly, GST X11α (region 163–315) binds efficiently to the Munc-18-1—Syntaxin complex (Fig. 1C). Together, these data show that mLin-2/CASK and Munc-18-1 proteins bind to the X11α N terminus on two different sites.

The first 320 amino acids of mLin-2/CASK encompassing the CKII domain directly binds to full-length X11α by overlay assay (Borg et al., 1998a). We used this assay to show that this region interacts with the X11α (region 373–436) peptide (Fig. 2A). Binding of GST mLin-2/CASK (region 1–320) to X11α was inhibited at a concentration of 250 nM soluble His-mLin-2/CASK (region 1–320), whereas 5 μM control protein did not affect the binding (Fig. 2B). These data allow us to conclude that mLin-2/CASK (region 1–320) binds tightly to the 63 amino acid peptide found in the X11α N terminus.

The mLin-2/CASK region 1–320 contains a CKII domain, followed by a calmodulin binding site (residues 294–320). We asked whether the peptide (region 294–320) was involved in the binding with X11α. Various mLin-2/CASK GST fusion proteins were used to precipitate myc-tagged X11α expressed in 293 cells (Fig. 3A). Bound proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and revealed with anti-myc antibody. GST mLin-2/CASK (region 1–294) does not contain the calmodulin binding site but still binds very well to X11α (Fig. 3B). We also introduced deletions within the CKII domain of mLin-2/CASK; truncated CKII protein does not bind to X11α, suggesting that residues 1–294 are required for the proper folding and/or function of the domain (Fig. 3C).

Calmodulin binds to mLin-2/CASK and does not affect X11α—mLin-2/CASK interaction

Our next experiments aimed to determine the role of calmodulin in X11α—mLin-2/CASK association. Calmodulin-dependent kinases require the binding of Ca2+/calmodulin to activate their catalytic activity (Goldberg et al., 1996). It has been suggested that Ca2+/calmodulin binds to a GST mLin-2/CASK fusion protein (Hata et al., 1996). We used an in vitro binding assay to detect an interaction between the mLin-2/CASK (region 294–320) pep-
tide and purified calmodulin. Increasing amounts of calmodulin were incubated with immobilized GST mLin-2/CASK (regions 1–320 or 1–294) protein, and bound proteins were revealed with anti-T7 antibody, followed by HRP goat anti-mouse and chemiluminescence detection (right). The same procedure was performed to detect X11α in 293 cell lysate, except that soluble GST mLin-2/CASK (region 1–320) protein was used as a primary reagent and anti-GST antibody–HRP protein A as secondary reagents. Increasing concentrations of soluble His-mLin-2/CASK (region 1–320) were mixed with a fixed concentration of soluble GST mLin-2/CASK (region 1–320) protein to compete for binding to X11α. A soluble His-X11α PDZ containing the two X11α PDZ domains was used as a negative control.

**A region of mLin-2/CASK encompassing the GK domain binds to X11α**

We found that the mLin-2/CASK CKII domain is crucial for in vivo interaction with X11α. Indeed, a mLin-2/CASK protein containing only the CKII and PDZ domains (region 1–612) coimmunoprecipitates with X11α, and this binding is conferred by the CKII domain (Borg et al., 1998a). However, in vitro binding assays led us to consider also an interaction between X11α and the second half of mLin-2/CASK containing an SH3 and GK domain (region 578–897). In Figure 5A, we show that the GST mLin-2/CASK (regions 1–612 and 578–897) fusion proteins bind to X11α in a specific manner because neither X11β nor X11γ can bind to these fusion proteins (Fig. 5A; data not shown). Binding of X11α to mLin-2/CASK (region 578–897) was reproducibly weaker than binding to mLin-2/CASK (region 1–612). This interaction is direct because soluble GST mLin-2/CASK (region 578–897) binds to X11α in an overlay assay (Fig. 5B). Whereas GST mLin-2/CASK (region 1–320) detects X11α in brain lysate, no signal is obtained with GST mLin-2/CASK (region 578–897), suggesting again a low-affinity interaction. The SH3 domain of mLin-2/CASK does not participate in this interaction because GST mLin-2/CASK (region 658–897) is sufficient for binding to X11α (Fig. 5C). We were also able to exclude a role for the lysine-rich band 4.1 binding regions (Cohen et al., 1998) because an mLin-2/CASK GST construct containing amino acids 700–897 was also able to bind X11α (results not shown). Thus, both the CKII and the GK domain containing regions in mLin-2/CASK bind to X11α. This dual contact probably increases the interaction between the two partners.
Figure 4. Calmodulin binds to mLin-2/CASK but does not affect X11α–mLin-2 interaction. A, GST and GST mLin-2/CASK (regions 1–320 and 1–294) were incubated with 0, 2, or 10 μg of calmodulin in the presence of 0.1 mM Ca$^{2+}$ or 1 mM EGTA (asterisk). After washing, bound calmodulin was resolved on SDS-PAGE, transferred to nitrocellulose, and detected with anti-calmodulin antibody. B, Lysates from untransfected A-172 cells were immunoprecipitated with preimmune or immune anti-mLin-2/CASK antibodies, and bound proteins were resolved on SDS-PAGE and transferred to nitrocellulose. Proteins were successively revealed with anti-mLin-2/CASK (top) and anti-calmodulin (bottom) antibodies. C, GST mLin-2/CASK (region 1–320) fusion protein immobilized on glutathione beads was incubated with Ca$^{2+}$/calmodulin, and then lysate with (+X11α) or without (−X11α) myc-tagged X11α was added. Bound calmodulin and X11α was then assessed using immunoblotting.

Localization of X11α in the brain
To examine the expression pattern of X11α, protein extracts from various organs were run on SDS-PAGE, transferred to nitrocellulose, and revealed with anti-X11 antibody. X11α is only expressed in the brain, whereas mLin-2/CASK is present in all tissues (Fig. 6A). Previous analyses have shown that X11α is detected in the cerebellum and hippocampus in mouse brain by in situ hybridization analysis (Duclos et al., 1993). We obtained similar results with a human X11α probe in rat brains (Fig. 6E). In addition, strong labeling was observed in the olfactory system, the piriform and entorhinal cortex, the suprachiasmatic nucleus of the hypothalamus, the substantia nigra, and other mesencephalic areas. In control experiments using a sense RNA probe, no signal was observed (data not shown). We performed immunostaining with anti-X11α antibody on rat brain sections to document the localization of the protein. No signal was detected when antibody was preincubated with the immunogen (Fig. 6, compare B, D). The substantia nigra is strongly stained by anti-X11α antibody, and this expression correlates with a positive signal by in situ hybridization (Fig. 6E). High magnification of neurons in substantia nigra shows a diffuse staining in intracellular compartments, with exclusion of nuclei (Fig. 6C).

Several rat brain sections were stained to study X11α expression in more detail. X11α-positive immunostaining was observed in several nuclei throughout the rat brain. Within the telencephalon, the main olfactory bulb exhibited heavy staining, especially in the mitral and external plexiform layers. In addition, some staining was observed in the glomerular layer (Fig. 7A,B). The internal granule cell layer was basically unstained. The piriform cortex exhibited a large number of intensely stained neurons, which were continuous through the entorhinal cortex (Fig. 7C,D). Throughout the cortex, layer V was stained with X11α neurons (Fig. 7G). In addition, layers II–IV also exhibited weak staining, whereas layer I was devoid of staining (data not shown). Within the striatum, X11α-immunopositive medium-sized neurons were scattered in distribution (Fig. 7E). The striatal neurons exhibited a characteristic punctuate labeling (Fig. 7F). Furthermore, scattered stained neurons were observed in the septum, nucleus accumbens, substantia innominata, and olfactory tubercle. In the nucleus of the diagonal band and the medial preoptic nucleus, numerous positive cells of various sizes and intensity of staining were observed. Few intensely stained and scattered neurons were observed in the hippocampus. In addition, the dentate gyrus
Several amygdaloid nuclei contained large and intensely X11α-stained neurons. Caudally in the diencephalon, many hypothalamic and thalamic nuclei exhibited positive-stained neurons of various sizes and intensity. For instance, a dense group of heavily stained neurons was present in the supraoptic nucleus and dorsally in the habenula. Intense staining was also present in the median eminence. Within the mesencephalon, scattered positive neurons were observed in the superior and inferior colliculus. A large number of heavily stained neurons, together with a dense fiber network, was present in the substantia nigra, gigantocellular reticular nucleus, and red nucleus (Fig. 7H). There was also a moderate staining in neurons of the dorsal raphe, as well as in the dorsal cochlear nucleus. Many nuclei within the caudal-most region of the brain corresponding to pons and medulla exhibited a large number of intensely stained neurons and fibers. Cells within these region exhibited a characteristic punctuate staining of the soma, axons, and dendrites, whereas in most cases, the nucleus was devoid of staining. The cerebellum exhibited a moderate staining, with intense staining of the Purkinje cells (data not shown).

**Localization of X11α–mLin-2 in NT2 neurons**

In our next series of studies, we wanted to determine where these proteins might interact within cells. To examine the localization...
of these proteins within neurons, we performed immunostaining of human NT2 neurons. NT2 teratocarcinoma cells form neurons when differentiated with retinoic acid (Pleasure and Lee, 1993). X11α is expressed in the differentiated NT2 cells but not in the stem cells (Fig. 8A). Staining of cells with anti-X11α antibodies indicates that the endogenous protein is localized in the cytosol and in a perinuclear region (Figs. 8A, B). A similar localization was seen when a myc-tagged X11α protein was expressed in PC12

Figure 7. X11α immunostaining in the rat brain. A, Photomicrograph of a coronal section through the main olfactory bulb (MOB) immunostained for X11α. Scale bar, 15 μm. B, High-magnification photomicrograph of the main olfactory bulb showing dense staining of the mitral cell layer. Note the intensely labeled dendrites extending to the glomerular layer. Scale bar, 40 μm. C, Coronal section through the piriform cortex (PO). Scale bar, 230 μm. D, High-magnification photomicrograph of the intensely stained pyramidal cells of layer 2 of the piriform. Scale bar, 40 μm. E, X11α-immunostained section exhibiting scattered cells in the striatum. Scale bar, 230 μm. F, Detail of a X11α-positive neuron in the striatum. Note the punctuate labeling along the axon. Scale bar, 40 μm. G, Coronal section through the cortex stained with X11α antibody. The staining is most prominent in layer V. Scale bar, 100 μm. H, High-magnification photomicrograph at the level of the substantia nigra with cells strongly stained for X11α. Note the lack of nuclear staining and the punctuate labeling. Scale bar, 40 μm.
cells (data not shown). Lin-10 has also been localized to the perinuclear region in C. elegans neurons (Rongo et al., 1998). mLin-2/CASK localized to the same regions in cells (Fig. 8B). The perinuclear region represents a component of the wheat germ ag-stained regions (results not shown). Furthermore, X11α colocalizes with giantin, suggesting that X11α and mLin-2/CASK are in a fraction of the golgi apparatus (Linstedt and Hausi, 1993). Interestingly, APP, an X11α partner, is also predominately located in the golgi and trans-golgi network in neurons (Caporaso et al., 1994).

**DISCUSSION**

This study further delineates the interaction between X11α and mLin-2/CASK, two proteins involved in receptor localization in neurons (Borg et al., 1998; Butz et al., 1998; Kaech et al., 1998; Rongo et al., 1998). We show that a 63 amino acid peptide found in X11α interacts with the mLin-2/CASK CKII domain. Munc-18-1, another X11α partner, binds to a different location in the X11α N terminus. Genetic analyses have suggested that the Lin-2 CKII domain acts like a protein–protein interaction domain rather than a kinase (Hoskins et al., 1996). A similar conclusion is drawn from our biochemical data with the mLin-2/CASK CKII domain. Calmodulin is a cellular calcium sensor for many enzymes and regulates ion channels, cell cycle, and cytoskeletal organization (James et al., 1995). Furthermore, previous studies have demonstrated a role for calmodulin as a negative regulator of protein–protein interactions (Wyszynski et al., 1997). In contrast, we show that calmodulin binding does not affect X11α–mLin-2 interaction. Deletion analysis of the mLin-2/CASK CKII domain has demonstrated that the integrity of the CKII domain is required for proper binding to X11α. We also describe an interaction between the mLin-2/CASK C terminus containing a GK domain and X11α. This interaction is weaker than the interaction between the CKII domain of mLin-2/CASK and X11α. Accordingly, we feel that this interaction involving the GK domain may increase the avidity of X11 with mLin-2/CASK but cannot be solely responsible for the interaction. In C. elegans, a lin-2 transgene, mutated to produce a protein with a catalytically inactive GK domain, is able to rescue the vulvaless phenotype caused by lin-2 mutations (Hoskins et al., 1996). Furthermore, the GK domain of PSD-95 and synaptic scaffolding molecule interacts with proteins found in postsynaptic densities (Kim et al., 1997; Hirao et al., 1998). Together, these data argue that the GK domain of MAGUK proteins acts as a protein–protein interaction domain.

APP, neurexins, and syndecans bind to the PTB and PDZ domains found in the X11α–mLin-2 complex (Borg et al., 1996, 1998a; Hata et al., 1996; Cohen et al., 1998; Hsueh et al., 1998). Additionally, it has been shown that mLin-7 binds tightly to mLin-2/CASK (Borg et al., 1998a; Butz et al., 1998; Kaech et al., 1998). Considering that the PDZ domains of mLin-7 and X11α and the SH3 domain of mLin-2/CASK are also available for interactions, further studies will certainly increase the number of interactors found in this complex. Such complexity is common for PDZ domain proteins. For example, in Drosophila, inactivation no afterpotential D (INAD), a PDZ protein, contacts multiple partners (phospholipase C, calmodulin, rhodopsin, and TRP ion channel) important in phototransduction (Xu et al., 1998). In worms, the Lin-10–Lin-2–Lin-7 heterotrimeric complex targets Let-23 to the basolateral epithelium in vulva (Kaech et al., 1998). In mammals, no precise role has been assigned to the complex, but a role in receptor localization is probable. Munc-18-1–Syntaxin is a brain-specific plasma membrane complex involved in the docking of vesicles during exocytosis (Hata et al., 1993). A possible scenario is that the X11α–mLin-2–mLin-7 complex binds to Munc-18-1–Syntaxin at the plasma membrane to deliver receptors such as APP, neurexins, and syndecans. We have tried to detect an in vivo interaction between the heterotrimeric complex and Munc-18-1–syntaxin. Although we could easily coimmunoprecipitate X11α, mLin-2/CASK, and mLin-7 from mouse brain extracts, our attempts to demonstrate a coimmunoprecipitation with Munc-18-1–Syntaxin were unsuccessful. However, other groups have detected a complex containing X11α/Mint-1 and Munc-18-1 (Okamoto and Sudhof, 1997).

**In situ** hybridization data show that X11α is present in the hippocampus, cerebral cortex, anterior thalamic nuclei, and cerebellum. In addition, positive signal was observed in the olfactory bulb, the piriform cortex, hypothalamus, and other thalamic areas, as well as numerous brainstem areas. Immunohistochemical data demonstrate that, in general, protein and mRNA follow a similar pattern for X11α distribution. However, in some areas, such as the hippocampus, mRNA expression was highly abundant, but protein levels appeared to be relatively low. These differences may be explained by a high level of mRNA expression versus a
CASK PDZ domain.

with X11 PTB domains, whereas the mLin-2/CASK–mLin-7 complex only interacts with X11α. The Munc-18-1–Syntaxin neuronal complex interacts with the neuronal X11 species. Neuroxins and syndecans binds to the mLin-2/CASK PDZ domain.

low translational rate and/or a high proteolytic rate. mLin-2/CASK is ubiquitously expressed, with a predominant expression in the brain (Hata et al., 1996; Cohen et al., 1998) (Fig. 6A). Like X11α, mLin-2/CASK is distributed in a punctate somatodendritic pattern in neurons. Many regions are positive for X11α and mLin-2/CASK expression. For example, cortical layer V pyramidal neurons and their apical dendrites present an overlapping staining. The same punctate nature of staining is seen in pyramidal cell dendrites, which suggests a synaptic localization of the two proteins. X11α and mLin-2/CASK are also found in neurons of the thalamus and in Purkinje cells of the cerebellum (Hsueh et al., 1998). However, mLin-2/CASK has not been described in some other areas, such as the hypothalamus or brainstem nuclei, where X11α is expressed. A more complete study of the distribution of mLin-2/CASK may reveal a wider distribution than previously described. Unfortunately, the anti-mLin-2/CASK that we produced and the commercially available anti-mLin-2/CASK antibodies did not give any specific signal in rat brain immunohistochemistry. We find this complex in neurons, where it is likely to play an important role in the localization of proteins to presynaptic or postsynaptic sites. In human neurons, X11α is found in the cytosol and in a component of the golgi network. Although the significance of this localization is presently unclear, we speculate that X11α in these compartments is required for the proper targeting of receptors. Although we could not localize mLin-2/CASK to specific structures other than the golgi in NT2 neurons, recent studies have also localized the protein to synapses and basolateral membranes of epithelial cells (Cohen et al., 1998; Hsueh et al., 1998).

The X11β gene is also expressed in the brain, and the encoded protein is functionally related to X11α because it binds to APP and Munc-18-1–Syntaxin (Okamoto and Sudhof, 1997; Borg et al., 1998b). The lack of binding to mLin-2/CASK probably creates functional differences with X11α. Finally, X11γ does not bind to mLin-2/CASK and Munc-18-1 but still binds to APP. In neurons, APP is associated with at least three different intracellular complexes containing X11 proteins (Fig. 9). An alteration in localization of APP or its retention in a subcellular compartment induced by X11α may explain the effects of X11α on the processing of APP (Borg et al., 1998b). Additionally, members of the Fe65 protein family bind to the cytoplasmic region of APP (Borg et al., 1996; Fiore et al., 1996; Guenette et al., 1996; Trommsdorff et al., 1998). These multiple complexes may play a role in normal and pathological metabolism of APP in neurons.

REFERENCES


Figure 9. The X11 protein family participates in multiple protein complexes in neurons. Schematic representation of the different proteins interacting with X11 proteins. X11α and X11β are highly expressed in brain, whereas X11γ is ubiquitously expressed. APP binds to all three X11 PTB domains, whereas the mLin-2/CASK–mLin-7 complex only interacts with X11α. The Munc-18-1–Syntaxin neuronal complex interacts with the neuronal X11 species. Neuroxins and syndecans binds to the mLin-2/CASK PDZ domain.