Identification of an Evolutionarily Conserved Heterotrimeric Protein Complex Involved in Protein Targeting*

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In Caenorhabditis elegans, lin-2, lin-7, and lin-10 genetically interact to control the trafficking of the Let-23 growth factor receptor to the basolateral surface of body epithelia. The human homologue of the lin-10 gene has recently been identified as a member of the X11 family. The X11 proteins contain one phosphotyrosine binding (PTB) and two PSD-95-Dlg/ZO-1 (PDZ) domains as well as an extended amino terminus. We have previously shown that the PTB domain of X11a (also known as Mint1) can bind to the amyloid precursor protein (APP) in a phosphotyrosine-independent fashion and can markedly inhibit the processing of APP to the amyloid β (Aβ) peptide. Here, we report that X11a directly binds to the mammalian homologue of Lin-2 (mLin-2), also known as CASK. This binding is mediated by direct interaction between the Calmodulin Kinase II (CKII)-like domain of mLin-2 and the amino terminus of X11a. Furthermore, we can detect direct interactions between mLin-2 and mammalian Lin-7 (mLin-7). In mouse brain, we have identified a heterotrimeric complex that contains mLin-2, mLin-7, and X11a and that is likely important for the localization of proteins in polarized cells. This complex may play an important role in the trafficking and processing of APP in neurons.

Protein-protein interactions are crucial for many cellular processes and are mediated by protein domains that are highly conserved in evolution (1). The PTB domain was first identified in Shc and IRS-1 (2–5) and subsequently found in a large number of proteins (6, 7). In these proteins, this domain binds to Asn-Pro-X-pTyr, a β turn motif found on activated growth factor receptors and other signaling molecules. After binding to growth factor receptors, both Shc and IRS-1 are themselves tyrosine-phosphorylated and coupled to downstream signaling molecules. Despite the name, there is strong evidence that some PTB domains bind to their target proteins in a phosphotyrosine-independent fashion (7). Perhaps this binding is best understood for the X11 protein where biochemical studies have demonstrated an interaction between the X11 PTB domain and APP (8, 9). Structural studies indicate that the X11 PTB domain binds a nonphosphorylated beta turn motif on APP (10). Evidence has also been provided for phosphotyrosine-independent interactions of the Numb PTB domain (11–13). The finding that PTB domains can bind to their target peptides in a phosphotyrosine-independent fashion indicates that these domains can be involved in diverse cellular functions, not just signaling downstream of tyrosine kinases.

PTB domains are often found in combination with other protein-protein interaction domains. For example, Shc has both an SH2 domain and a PTB domain, whereas X11 proteins have two PDZ domains in addition to a PTB domain. PDZ domains have been shown to bind to the carboxyl terminus of proteins by wrapping around the extreme carboxyl-terminal residues (14, 15). PDZ domain proteins such as PSD-95 have been demonstrated to play a role in receptor and channel clustering at synaptic junctions (16–19). PDZ domains have also been implicated as being important in protein targeting to specific membrane surfaces. In Caenorhabditis elegans, the lin-2, lin-7, and lin-10 genes are important for the proper localization of the Let-23 growth factor receptor to the basolateral side of the body wall epithelium (20–22). Let-23 is related to mammalian EGF receptors and binds to the lin-3 gene product, a protein related to TGF-α (23, 24). Lin-3 is released by the anchor cell of the gonad and induces the epithelium to form a vulva by activating Let-23. Mutations in lin-2, lin-7, or lin-10 impair vulval formation likely because of mislocalization of the Let-23 receptor and its inability to efficiently bind Lin-3 (22). Originally Lin-10 was felt to be a protein unrelated to previously identified proteins (25). However recent work has reassigned the product of the lin-10 gene as a homologue of the X11 family of proteins.

In this work, we define a protein complex in mammalian brain that contains X11a, mLin-2, and mLin-7. Thus, these protein interact both biochemically and genetically and likely control protein targeting in an evolutionarily conserved fashion.

EXPERIMENTAL PROCEDURES

Antibodies—Anti-MyC 9E10 (Oncogene Research Products) and anti-FA 12CA5 (Boehringer Mannheim) monoclonal antibodies were used for immunoprecipitation and immunoblotting. Anti-PSD-95 monoclonal antibody is from Upstate Biotechnology. Anti-synaptin-1 monoclonal antibody is from Sigma. Polyclonal anti-mLin-2, anti-X11, and anti-mLin7 antibodies were prepared by injecting rabbits with the following main: PDZ, PSD-95-Dlg-ZO-1; APP, amyloid precursor protein; CKII, calmodulin-dependent kinase II; mLin-2, mammalian Lin-2; mLin-7, mammalian Lin-7; Aβ, amyloid beta; EST, expressed sequence tag; GST, glutathione S-transferase; HA, hemagglutinin; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; GK, guanylate kinase; EGF, epidermal growth factor.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF070975.

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† The abbreviations used are: PTB, phosphotyrosine binding domain.
buffered saline and lysed in lysis buffer (50 mM HEPES, pH 7.5, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA) supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 mM aprotinin, and 10 μg/ml Streptokinase.

For immunoprecipitation, lysates of 293 cells transiently cotransfected with RK5-myc (vector), RK5-myc-mLin-2 (mLin-2), RK5-myc-mLin-2 (CKII + PDZ) or RK5-myc-mLin-2 (SH3 + GK) were incubated with anti-myc antibody bound to beads. After washing, immune complexes were resolved on a 10% SDS-PAGE, transferred to nitrocellulose, and revealed with anti-myc antibody bound to mouse brain fractions were subjected to overlay assay: lysate before depletion (lysate), lysate depleted of X11a (lysate post-IP anti-X11), proteins bound on pre-immune serum (IP control), or anti-X11 (IP anti-X11) antibodies. The membrane was probed with soluble GST-mLin-2 (CKII) protein, and bound proteins were revealed with anti-GST antibody followed by HRP-protein A and chemiluminescence detection. X11a is indicated by an arrow. No signal was detected with soluble GST protein (not shown). D, the same procedure was performed to detect myc-tagged X11a, X11g, X11y, and Lin-10 in 293 cell lysates with GST-mLin-2 (CKII) (top panel). The level of protein expression was detected by blotting with anti-myc antibody (bottom panel).
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**RESULTS AND DISCUSSION**

We have identified three members of the X11 protein family. X11α/Mint1 and X11β/Mint2 are primarily expressed in the central nervous system, whereas X11γ/Mint3 is more widely expressed (26). All X11 family members have conserved PTB and PDZ domains but divergent amino termini. Our previous work has detected an interaction between X11 family members and APP via the X11 PTB domain (8). This binding prolongs the half-life of APP and slows its processing to the pro-amyloidogenic Aβ peptide (26, 27). As X11α has multiple protein–protein interaction domains (Fig. 1A), we looked for proteins other than APP that could bind to X11α. In [35S]methionine-labeled A-172 cells, we were able to show that X11α coinmunoprecipitates with a 110-kDa protein. X11α binds to this 110-kDa protein via residues 163–436 in its amino terminus (Fig. 1B).

Recent evidence indicates that the X11 proteins have a close homologue in *C. elegans* encoded by the *lin-10* gene (2). In worms, the *lin-2* gene is linked to the *lin-10* gene and encodes a 110-kDa protein (Fig. 1A), a size compatible with the protein binding to the amino terminus of X11α. A rat homologue of *lin-2* encoding a neurexin-binding protein known as CASK has been identified (28). We identified the human *mlin-2/CASK* gene from the EST data base and constructed an mLin-2 protein fused to a myc epitope. Antibodies raised against mLin-2 recognized the 110-kDa protein bound to X11α (not shown). When 293 cells were transfected with X11α and myc-tagged mLin-2, we were able to coinmunoprecipitate the proteins. Deletions of mLin-2 show that the SH3 and GK domains are dispensable for this interaction (Fig. 2A). The binding site for the amino terminus of X11α mapped to the CKII-like domain. This CKII domain, when expressed as a GST fusion protein, could bind X11α in precipitation reactions and in Far Western blotting (Fig. 2B and C). This domain not only recognized X11α but also the *C. elegans* X11 homologue, Lin-10. In contrast the mLin-2 CKII domain did not recognize two other isoforms of X11, X11β and X11γ (Fig. 2D). This is consistent with the finding that, although X11α, X11β, and X11γ have conserved PTB and PDZ domains, they have divergent amino termini. Similarly, in 293 cells we could show that mLin-2 coinmunoprecipitates with X11α but not X11β or X11γ (results not shown). The kinase and calmodulin binding site of calmodulin kinase II α is 45% identical to the CKII domain of mLin-2 but
does not bind to X11α (result not shown). The CKII domain of mLin-2 does not appear to encode an active kinase (21, 28) but rather appears to function as a protein-protein interaction domain.

We examined the interaction of X11α and mLin-2 in mouse brain. We were able to show that antibodies to X11α were able to coimmunoprecipitate mLin-2 from mouse brain (Fig. 3A). We found both proteins in cytosolic and membrane fractions (Fig. 3B) where they coimmunoprecipitate (Fig. 3C). When we immunodepleted brain cytosolic fraction of X11α, we also removed a large amount of mLin-2 from the lysates (Fig. 3D), indicating that a significant fraction of X11α and mLin-2 are bound together. In worms, lin-7 mutants yield a similar phenotype as is seen with lin-2 and lin-10 mutants (22). In mouse brain, we were able to show that immunoprecipitation with anti-mLin-7 antibodies coimmunoprecipitiated X11α and mLin-2 proteins (Fig. 4A). GST-mLin-7 can bind mLin-2 but not X11α (results not shown), demonstrating that mLin-7 is bound to mLin-2, whereas mLin-2 is bound to X11α. We have mapped the site of interaction for mLin-7 to the region between the CKII and PDZ domain of mLin-2 (Fig. 4B). Conversely, we have mapped the binding site for mLin-2 to the amino-terminal half of Lin-7 (Fig. 4C).

In summary, we have identified a heterotrimeric complex in the brain that consists of X11α, mLin-2, and mLin-7. In worms, genetic analysis has shown that homologous proteins play a central role in targeting of the Let-23 receptor to the basolateral surface of epithelial cells (22). This process is mediated by the binding of the Lin-7 PDZ domain to Let-23 (20). However, we have found that none of the mammalian EGF-receptor family members (HER1 through HER4) binds to mLin-7.4 In neurons, the X11α-mLin-2-mLin-7 protein complexes may bind to specific target membranes sites via Munc-18–1, a protein that can bind syntaxins and possibly modulate trafficking and secretion (29). Once bound to membranes, X11α, mLin-2, and mLin-7 proteins either individually or in a complex may serve to localize or retain proteins at specific membrane sites. Recent studies have detected a fraction of mLin-2/CASK at neuronal synapses (30) as well as at the basolateral surface of epithelial cells (31). Because of the brain-specific expression of X11α, we can exclude a role for X11α in the basolateral localization of mLin-2 in mammalian epithelia. X11α, the only X11 species that we have detected in epithelium, does not interact with mLin-2. This suggests a possible divergence between worm and mammalian epithelia because, in worm epithelia, the X11a homologue, Lin-10, is crucial for basolateral targeting. The heterotrimeric complex contains several protein-protein interaction domains that would be useful to contact a large number of different proteins (30, 31). In addition, the presence of the PTB domain, a domain that can bind to beta turn motifs, might make X11 proteins particularly suitable for detecting traffick

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4 Straight, J.-P., Borg, and B. Margolis, unpublished observations.

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**Addendum**—While this manuscript was under review, additional studies of the X11α-mLin-2-mLin-7 complex in worms and mammals were published (Butz, S., Okamoto, M., and Sudhof, T. C. (1998) Cell 94, 773–782, and Kaech, S. M., Whitfield, C. W., and Kim, S. K. (1998) Cell 94, 761–771).

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