

The Neuronal Adaptor Protein X11 α Interacts with the Copper Chaperone for SOD1 and Regulates SOD1 Activity*

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The neuronal adaptor protein X11 α participates in the formation of multiprotein complexes and intracellular trafficking. It contains a series of discrete protein-protein interaction domains including two contiguous C-terminal PDZ domains. We used the yeast two-hybrid system to screen for proteins that interact with the PDZ domains of human X11 α , and we isolated a clone encoding domains II and III of the copper chaperone for Cu,Zn-superoxide dismutase-1 (CCS). The X11 α /CCS interaction was confirmed in coimmunoprecipitation studies plus glutathione S-transferase fusion protein pull-down assays and was shown to be mediated via PDZ2 of X11 α and a sequence within the carboxyl terminus of domain III of CCS. CCS delivers the copper cofactor to the antioxidant superoxide dismutase-1 (SOD1) enzyme and is required for its activity. Overexpression of X11 α inhibited SOD1 activity in transfected Chinese hamster ovary cells which suggests that X11 α binding to CCS is inhibitory to SOD1 activation. X11 α also interacts with another copper-binding protein found in neurons, the Alzheimer's disease amyloid precursor protein. Thus, X11 α may participate in copper homeostasis within neurons.

The X11s, also known as mints (munc18 interacting proteins 1–3), are a family of adaptor proteins with three members (α , β , and γ) encoded by separate genes on, respectively, human chromosomes 9, 15, and 19 (1–8). Expression of X11 α and X11 β is restricted to neurons, whereas X11 γ is ubiquitously expressed (1, 4, 6–9). The X11s diverge substantially in their N-terminal regions, but they all contain a centrally located phosphotyrosine binding domain (10), through which all three X11s bind to the cytoplasmic domain of the Alzheimer's disease

amyloid precursor protein (APP)¹ (2, 6, 7, 11, 12), and two contiguous C-terminal PDZ (PSD-95, *Drosophila* disks-large, ZO-1) domains (13). These, and a variety of other less well characterized protein-protein interaction regions (Fig. 1A), mediate the binding of the X11s to a number of proteins.

X11 α binds to the pre-synaptic adaptor protein CASK via a sequence preceding the phosphotyrosine binding domain (9, 14, 15). CASK also binds both Veli1 and members of the neurexin family of pre-synaptic membrane-spanning proteins (14, 16). The X11 α -CASK-Veli pre-synaptic complex is highly evolutionarily conserved and is found in orthologous form in *Caenorhabditis elegans* as the LIN-10-LIN-2-LIN-7 complex (17) that regulates basolateral sorting of the epidermal growth factor receptor LET-23 and maintains cell polarity (18, 19). X11 α also binds to the synaptic vesicle docking protein Munc-18 through an N-terminal region (3, 4, 14). Via its first PDZ domain (PDZ1), X11 α has been reported to bind both the kinesin superfamily motor protein KIF17 (20) and the C terminus of the N-type Ca²⁺ channel pore-forming α_{1B} -subunit, which in turn can bind the SH3 region of CASK (21). Finally, we have shown that X11 α binds, via both of its PDZ domains, to presenilin-1 and mediates interactions between presenilin-1 and APP (22). Thus, similar to several other PDZ-bearing proteins (for reviews see Refs. 13 and 23), X11 α appears to provide a framework, or scaffolding, for the assembly of multimolecular complexes and functions in the trafficking and sorting of proteins to different neuronal compartments.

The full complement of ligands that bind to the X11 proteins is not yet known. In this study, we demonstrate that, via its second PDZ domain (PDZ2), X11 α interacts with the copper chaperone for copper/zinc superoxide dismutase-1 (CCS). CCS delivers and inserts the copper cofactor into the antioxidant enzyme copper/zinc superoxide dismutase-1 (SOD1) and is required for its activation (24–28). We also demonstrate that overexpression of X11 α inhibits SOD1 activity in transfected cells, consistent with a role for X11 α in regulating CCS function and SOD1 activation.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid System—Interactive cloning experiments, including relevant controls, were performed as described previously (2). In brief, the sequence encoding the two human X11 α PDZ domains (PDZ1 and -2; X11 α amino acid sequence 649–837) was amplified by polymerase chain reaction (22), subcloned into the *EcoRI* site of the yeast “bait” vector pY3 (29), and used to screen a human brain cDNA library (CLONTECH). Yeast colonies were grown using selective media lacking

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¹ The abbreviations used are: APP, amyloid precursor protein; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; SOD1, superoxide dismutase-1; ALS, amyotrophic lateral sclerosis.

tryptophan, leucine, and histidine. Vigorously growing colonies were subjected to freeze-fracture β -galactosidase assays, and candidate library plasmids were rescued from positive colonies by transformation into *Escherichia coli* HB101. Brain library cDNA inserts were sequenced using a Cyclist Exo⁻ *Pfu* DNA sequencing kit (Stratagene).

Mammalian Expression Plasmids and Transfection—Chinese hamster ovary (CHO) cells and rat primary cortical neurones were cultured as described previously (8, 30). CHO cells were transfected using LipofectAMINE (Life Technologies, Inc.) as per the manufacturer's instructions. Full-length C-terminal Myc-tagged human X11 α was as described previously (22). The cDNA encoding human CCS (24) was subcloned into pCDNA3.1 (Invitrogen), and human SOD1 was expressed in pCIneo (Promega).

Antibodies—X11 α was detected using rabbit polyclonal antibodies raised against X11 α -(161–421) (22). The same immunogen was also used to generate mouse polyclonal antibodies. Antibody specificity was confirmed by competing out signals with immunogen (data not shown). Rabbit polyclonal antibodies raised against CCS residues 1–85 were used to detect CCS (26). SOD1 sheep polyclonal antibody was purchased from Calbiochem, and the 9E10 anti-Myc monoclonal antibody was purchased from Sigma.

Coimmunoprecipitation Studies—CHO cells were doubly or singly transfected as indicated with Myc-tagged X11 α , CCS, and SOD1. Transfected cells were harvested in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 5 μ g/ml leupeptin, 2 μ g/ml aprotinin, 5 μ g/ml pepstatin, and 0.25 mM phenylmethylsulfonyl fluoride) and incubated on ice for 20 min. In binding studies with transfected SOD1, the cell lysates also underwent one freeze-thaw cycle (26). Lysates were then centrifuged at 14,000 rpm for 10 min at 4 $^{\circ}$ C. For immunoprecipitation studies the supernatant was precleared with protein A-Sepharose beads (Sigma). Myc-tagged X11 α was immunoprecipitated from 500 μ g of total protein lysate using antibody 9E10. The antibody was captured using protein A-Sepharose beads that were then washed four times with ice-cold lysis buffer. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotting as described (8).

GST Fusion Protein Binding Assays—Glutathione *S*-transferase (GST) fusion proteins with X11 α PDZ1-(649–746), PDZ2-(742–837), and PDZ1 + 2-(649–837) were as described (22). The partial CCS clone (CCS-(48–274)) isolated in the library screen encoded the C-terminal 227 amino acids of CCS, incorporating the C-terminal 39 residues of domain I and all of domains II and III of CCS (Fig. 1B). Digestion of this clone with *Bgl*III released cDNAs encoding CCS-(48–241) and CCS-(243–274) that were subcloned into the *Bam*HI site of pGEX-5X-1 (Amersham Pharmacia Biotech). CCS-(48–241) encompasses all of domain II of CCS (amino acids 87–234), whereas CCS-(243–274) encodes the C-terminal 32 amino acids of CCS including the CXC copper-binding motif (31–33). Digestion of the library clone with *Eco*RI released the CCS-(48–274) insert, which was subcloned into pGEX-5X-1. Fusion proteins containing full-length CCS or the cytoplasmic domain of APP were as described previously (26, 34). Expression and purification of GST fusion proteins, plus their use as ligands in pull-down binding assays, were essentially as described (22). The immunoprecipitation and GST fusion assays to confirm the X11 α and CCS interaction were performed three times with similar results.

Indirect Immunofluorescence—Transfected CHO cells and rat neuronal cultures were fixed and prepared for immunofluorescence as described (8, 22, 34). X11 α was detected in transfected CHO cells using the 9E10 antibody to the Myc tag; endogenous X11 α in primary rat cortical neurons was detected with the mouse X11 α antiserum. The rabbit antiserum was used to detect CCS in both transfected CHO cells and in neurons. Antibodies were visualized by goat anti-mouse Igs coupled to Texas Red and goat anti-rabbit Igs coupled to Oregon Green (Molecular Probes).

SOD1 Activity Assays—CHO cells were transfected with 2.5 μ g of SOD1, X11 α , and CCS DNA as indicated; transfections receiving only one or two plasmids were balanced with empty vector such that all received the same total amount of DNA. Transfected cells were washed twice with PBS, harvested by scraping into cold PBS, and then centrifuged at 10,000 \times *g* for 5 min at 4 $^{\circ}$ C. The pelleted cells were resuspended and lysed in water by a freeze-thaw cycle as described (35), centrifuged at 10,000 \times *g* for 5 min, and the supernatant collected. A sample of each supernatant was removed for SDS-PAGE and immunoblot analyses; the remainder was adjusted to 0.125 M Tris chloride, pH 6.8, 20% (v/v) glycerol, 0.025% bromophenol blue, and 0.1% Nonidet P-40 (35). 30 μ g of protein from each sample was run on 10% nondenaturing polyacrylamide gels, and SOD1 activities were determined by nitro blue tetrazolium in-gel staining assays as described (36). 1 μ g of protein from each supernatant sample was analyzed by immunoblotting to deter-

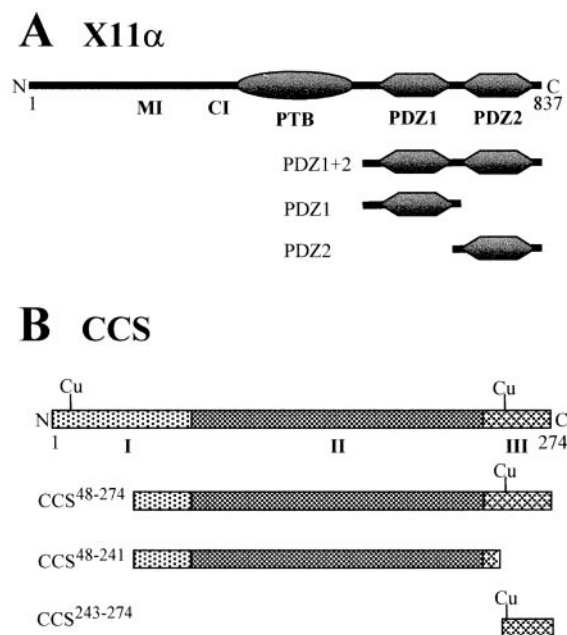


FIG. 1. Schematic representation of human X11 α and CCS. A, structure of human X11 α . MI, Munc-18 interacting region; CI, CASK interacting region; PTB, phosphotyrosine binding domain; PDZ, PSD-95, *Drosophila* disks-large, ZO-1 domain. The sequences used in this study encompassing PDZ1, PDZ2, and PDZ1 + 2 are indicated. B, structure of CCS depicting the location of domains I–III and the copper binding regions of CCS. The sequences identified and used in this study are indicated as CCS-(48–241), CCS-(243–274), and CCS-(48–274) as described in the text.

mine the expression of SOD1, X11 α , and CCS. Protein quantities were assayed using Bradford reagent (Sigma) as per the manufacturer's instructions. SOD1 activity assay gels and immunoblots were further analyzed by pixel densitometry using a Bio-Rad GS710 scanner and Quantity 1 software to obtain the ratio of SOD1 activity to expressed protein level for each sample (35). Within each set of transfections, this ratio was set at 100% for the SOD1-only transfected cells, and the remaining activities were expressed relative to this. Data from three separate sets of transfections were analyzed together using the Kruskal-Wallis one-way analysis of variance.

RESULTS

X11 α Binds to CCS—To identify binding partners for the X11 α PDZ1 + 2 region, we used the yeast two-hybrid system to screen a human brain cDNA library. A cDNA encoding the C-terminal 227 amino acids of CCS (CCS-(48–274)) was isolated (Fig. 1B). To investigate if the full-length proteins could interact in mammalian cells, immunoprecipitation experiments were performed from X11 α , CCS, and X11 α /CCS cotransfected CHO cells. X11 α was immunoprecipitated using the 9E10 antibody to the Myc tag. CCS was present only in immunoprecipitates obtained from X11 α /CCS-cotransfected cells but not X11 α or CCS alone transfected cells (Fig. 2A). Thus CCS coimmunoprecipitates with X11 α from transfected CHO cells.

Human CCS is comprised of three functional domains (31, 33). The N-terminal domain I (amino acids 1–86) is homologous to the secretory pathway copper chaperone HAH1 and its yeast ortholog Atx1p (37); the central domain II is ~50% identical to SOD1 (24); and the C-terminal domain III is unique to CCS (Fig. 1B). As all of domain II of CCS was encoded in the CCS cDNA isolated in the yeast two-hybrid screen, this raised the possibility that X11 α might also interact with SOD1. However, under similar experimental conditions as above, SOD1 did not coimmunoprecipitate with X11 α (Fig. 2B).

X11 α Binding to CCS Is Mediated by PDZ2 and Sequences within CCS Domain III—To map further the regions required

for binding of X11 α with CCS, we used GST fusion protein binding assays. GST fusion proteins containing X11 α PDZ1, PDZ2, and PDZ1 + 2 were used as baits in pull-down assays

from CCS-transfected CHO cells. CCS strongly interacted with PDZ2 and PDZ1 + 2, whereas no interaction could be detected between CCS and PDZ1 or GST alone (Fig. 3A). Since domain II of CCS is homologous to SOD1, we also tested whether SOD1 could bind to the X11 α PDZ domain fusion proteins. No interaction between any of the X11 α PDZ domains and SOD1 was detected (Fig. 3A), which is consistent with the immunoprecipitation experiments (Fig. 2B).

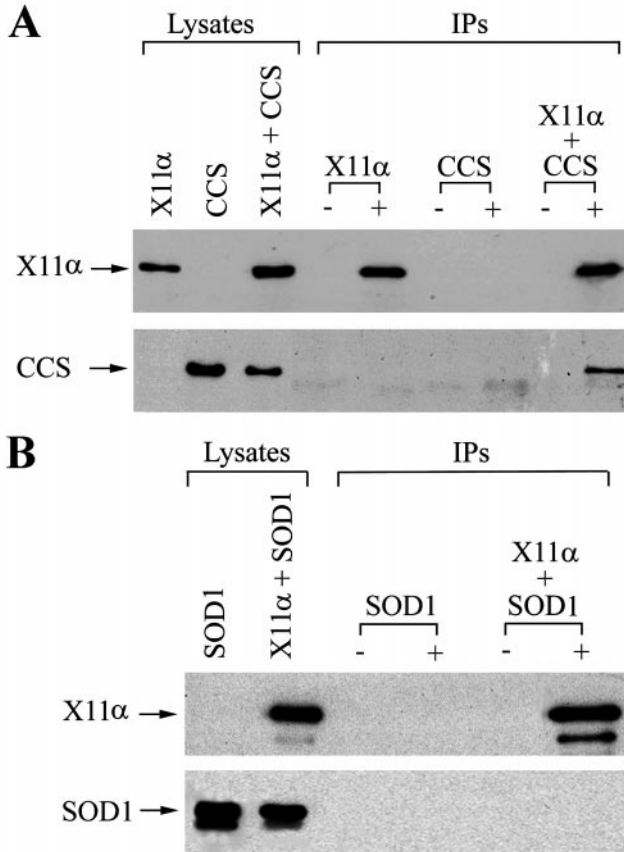


FIG. 2. X11 α interacts with CCS. *A*, immunoblot analysis of lysates and immunoprecipitates (*IPs*) from CHO cells transfected with Myc-tagged X11 α , CCS, or X11 α + CCS. X11 α was immunoprecipitated with 9E10 monoclonal antibody to the Myc tag, and the samples were analyzed by SDS-PAGE and immunoblotting with X11 α or CCS antisera. Immunoprecipitations were performed with (+) and without (-) 9E10 primary antibody. The lower level of CCS protein seen in cells cotransfected with X11 α in this particular experiment was not a consistent feature. *B*, immunoprecipitation studies of CHO cells transfected either individually or in combination with Myc-tagged X11 α and wild-type SOD1. X11 α was immunoprecipitated with the 9E10 antibody, and the samples were probed with antibodies to X11 α or SOD1.

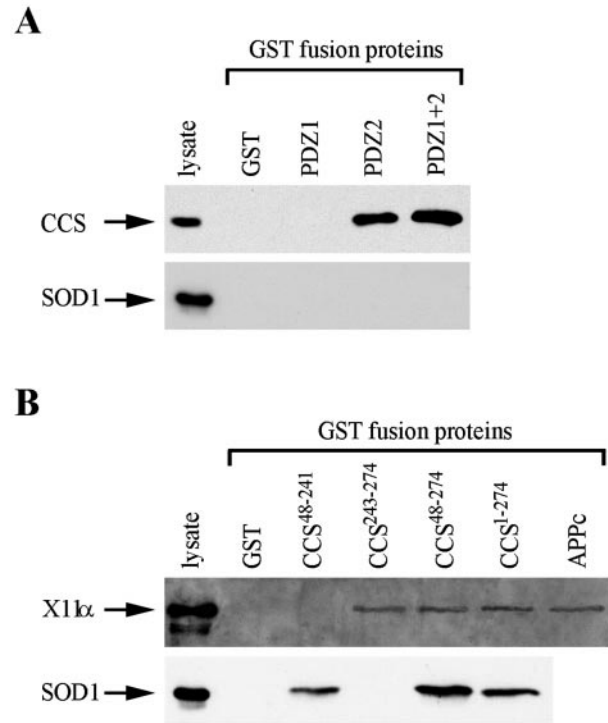
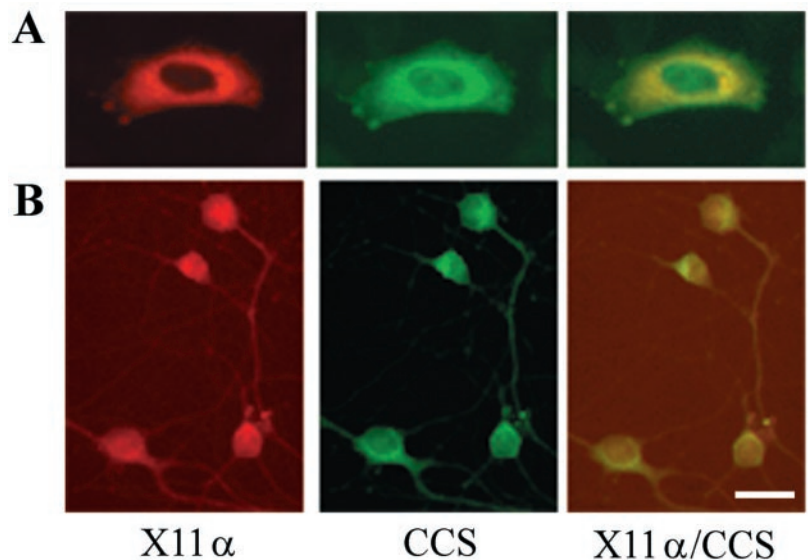


FIG. 3. X11 α PDZ2 interacts with sequences within domain III of CCS. *A*, GST and GST-X11 α PDZ domain fusion proteins were used as baits to probe for interactions with CCS and SOD1 from transfected CHO cell lysates. X11 α PDZ1 + 2 and PDZ2, but not PDZ1 or GST only, bound to CCS (*upper panel*). None of the baits interacted with SOD1 (*lower panel*). *B*, GST and GST-CCS fusion proteins were used as baits to probe for interactions with X11 α or SOD1 (*lower panel*). Full-length CCS (CCS-(1-274)) and CCS baits CCS-(243-274) and CCS-(48-274), but not CCS-(48-241) or GST alone, bound X11 α (*upper panel*). The C terminus of APP (APPc) also bound X11 α (*upper panel*). CCS baits CCS-(48-241), CCS-(48-274), and CCS-(1-274), but not CCS-(243-274) or GST alone, bound SOD1 (*lower panel*).

FIG. 4. Subcellular distribution of X11 α and CCS. *A*, double immunofluorescence in CHO cells cotransfected with Myc-tagged X11 α and CCS. X11 α was detected using the 9E10 antibody to the Myc tag, and CCS was detected using CCS rabbit antiserum. *B*, double immunofluorescence of rat primary cortical neurons with murine X11 α polyclonal antiserum and CCS rabbit antiserum. In the overlaid images (X11 α /CCS) yellow indicates regions of overlapping expression. Scale bar, 10 μ m.



In a complementary series of experiments, we used GST fusion proteins containing different domains of CCS in pull-down experiments from X11 α -transfected CHO cells. X11 α bound to full-length CCS, CCS-(48–274), which includes both domains II and III and replicates the results of the yeast two-hybrid screen, and also to CCS-(243–274) (*i.e.* the C-terminal 32 amino acids of domain III) but not to CCS-(48–241). GST alone did not bind X11 α (Fig. 3B). Thus sequences within domain III of CCS mediate the interaction with X11 α . The strength of the signals obtained using these CCS baits was generally weaker than those with the X11 α baits. However, it is notable that in these experiments the strength of signals obtained with the CCS baits was similar to that obtained with a GST bait containing the C-terminal 47-amino acid cytoplasmic domain of APP (Fig. 3B), which is known to bind to all X11 proteins (2, 6, 7, 11, 12). We also tested whether the CCS baits would interact with SOD1 in these pull-down assays. Only sequences that included domain II bound SOD1, a finding consistent with previous reports (26) (Fig. 3B). Taken together, these binding studies indicate that the interaction between human X11 α and CCS is mediated by the second PDZ domain of X11 α and sequences within domain III of CCS.

Cellular Distribution of X11 α and CCS in Cotransfected CHO Cells and in Rat Primary Cortical Neurons—Immunocytochemical labeling of X11 α /CCS cotransfected CHO cells revealed that both proteins had overlapping distribution patterns, particularly in the perinuclear region (Fig. 4A). X11 α and CCS also displayed an overlapping distribution pattern in rat primary cortical neurons where staining for both proteins was particularly pronounced in cell bodies with weaker labeling of neurites (Fig. 4B). These observations are consistent with previous reports that X11 α is present in cell bodies, possibly including the Golgi apparatus and to a lesser extent in axons, dendrites, and synapses (9, 38), and that CCS is enriched in neuronal cell bodies (39).

X11 α Inhibits SOD1 Enzymatic Activity—CCS delivers the metal ion copper cofactor to SOD1 (24–28), and copper is required for SOD1 activity (40). We therefore tested whether expression of X11 α could influence SOD1 activity. SOD1 activity assays were performed on mock-transfected CHO cells, and CHO cells transfected with SOD1, SOD1 + X11 α , SOD1 + CCS, and SOD1 + CCS + X11 α (Fig. 5A). Immunoblot analyses demonstrated that similar levels of transfected SOD1 were expressed in the different transfections (Fig. 5B). However, SOD1 activity was significantly reduced in CHO cells expressing X11 α (Kruskal-Wallis $\chi^2 = 8.74$, D.F. = 3, $p = 0.03$; Fig. 5, A and C). This included cells that were not cotransfected with CCS (Fig. 5A, track 3) which demonstrates inhibition of the action of endogenous CCS by X11 α .

DISCUSSION

In the present study we have demonstrated that human X11 α interacts with CCS in a variety of biochemical assays and that X11 α and CCS display overlapping subcellular distribution patterns in both neurons and transfected CHO cells. The interaction between X11 α and CCS is mediated via the second PDZ domain of X11 α and sequences within the C-terminal 32 amino acids of CCS. CCS is the first reported ligand specific for PDZ2 of X11 α . The prototypical PDZ domain binding sequence is the C-terminal motif X(S/T)X(V/I/L). It is now apparent, however, that PDZ domains bind to a much broader range of C-terminal sequences (for example see Ref. 41) and can also bind to internal sequences as well as to other PDZ domains (13, 23, 42). It is thus possible that PDZ2 of X11 α binds to the C terminus of CCS (*i.e.* PAHL) or alternatively to an internal sequence within the C-terminal 32 amino acids of domain III.

The anti-oxidant enzyme SOD1 catalyzes the disproportion-

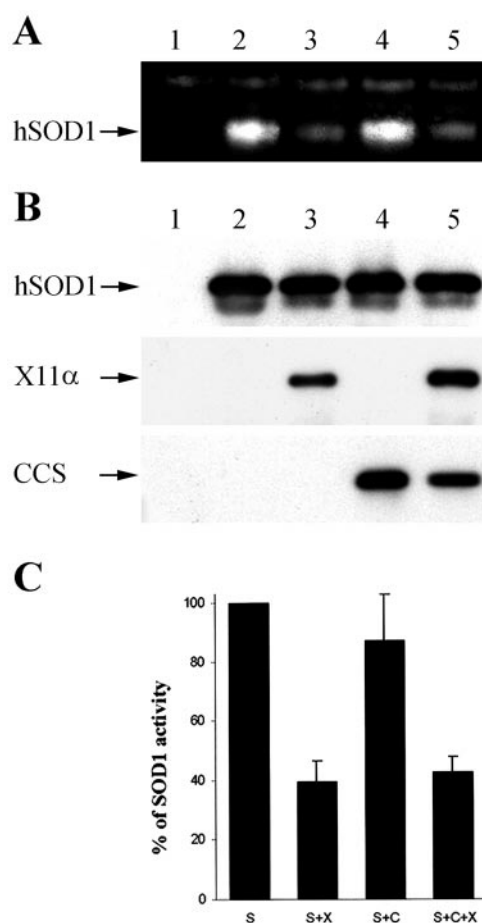


FIG. 5. X11 α down-regulates SOD1 activity. *A*, *in situ* nondenaturing gel assay of SOD1 activity in lysates from CHO cells, either mock-transfected (lane 1) or transfected with SOD1 (lane 2), SOD1 + X11 α (lane 3), SOD1 + CCS (lane 4) or SOD1 + X11 α + CCS (lane 5); human SOD1 homodimer (*hSOD1*), is indicated by an arrow. *B*, immunoblot analyses with antisera to detect SOD1, X11 α , and CCS in equivalent amounts of lysate from the same transfected cells as depicted in *A*. Extended exposure of the blots revealed the presence of endogenous CHO cell SOD1 but not CCS in the samples. We attribute this absence of signal for CCS to a failure of our antibody to cross-react with the CHO cell species. Indeed, transfected SOD1 displays significant activity in CHO cells in the absence of transfected CCS (*A*, lane 2) which demonstrates activation by endogenous CCS. The slight variation in the levels of X11 α and CCS seen in SOD1 + X11 α + CCS-transfected cells compared with cells transfected with SOD1 + X11 α and SOD1 + CCS was not a consistent feature of these experiments. *C*, relative activities of SOD1 in the presence of X11 α and/or CCS were calculated following quantification of SOD activities and expression levels. The data shown are the mean (S.D.) of three sets of experiments. *S*, SOD1; *S + X*, SOD1 + X11 α ; *S + C*, SOD1 + CCS; *S + C + X*, SOD1 + X11 α + CCS. The activities are presented as a percentage of activity found in SOD1 only (*S*) transfected cell lysates.

ation of superoxide anions through redox cycling of the bound copper ion in each monomer (40). Copper is delivered to SOD1 via CCS, and CCS is thus required for SOD1 activity (24, 25, 27, 28, 43). CCS comprises three domains as follows: an N-terminal domain I that is homologous to the Atx1p metallochaperone and that is involved in the recruitment of copper; a central domain II that exhibits homology to SOD1 and that facilitates the interaction of CCS with SOD1; and a C-terminal domain III that also binds copper but that additionally is involved in the interaction with SOD1 (26, 31, 44, 45). We mapped the X11 α -binding site to the C-terminal 32 amino acids of CCS, *i.e.* within domain III.

Overexpression of X11 α led to a down-regulation of SOD1 activity in transfected CHO cells, and this was not due to an

effect of X11 α on expression of either SOD1 or CCS (Fig. 5B). These observations suggest that X11 α binding to CCS is inhibitory to CCS function. Recent studies have highlighted the crucial importance of domain III in the delivery of copper from CCS to SOD1 (31, 44, 45). One suggestion is that domain III extends into the active site of SOD1 to facilitate insertion of copper into the enzyme (31, 44, 45). Thus, X11 α may inhibit SOD1 activity by binding to CCS domain III and disrupting this process in some way.

Altered copper homeostasis may be at least part of the pathogenic process in several neurodegenerative diseases (for reviews see Refs. 46 and 47). In particular, mutations in SOD1 are the causative genetic defect in some familial forms of amyotrophic lateral sclerosis (ALS) (48, 49). ALS mutant SOD1 proteins can bind CCS, and it has been suggested that mutant SOD1 may exert a toxic effect via altered cellular copper chemistry (25). In addition to binding CCS, X11 α interacts with another copper-binding protein, APP, mutations in which can cause Alzheimer's disease (see for review Ref. 50). APP has a conserved copper binding region in its ectodomain (51) and may play a role in the transport of copper to different cellular compartments (46). APP has been shown to reduce Cu(II) to Cu(I) upon binding Cu(II) and may thereby mediate copper-induced toxicity and oxidative stress in neurons (52–54). Thus, through its interactions with both CCS and APP, X11 α may play a general role in copper homeostasis within neurons. Defective copper metabolism, perhaps involving X11 α , may be mechanistic in both ALS and Alzheimer's disease.

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