The Coxsackievirus and Adenovirus Receptor (CAR) Forms a Complex with the PDZ Domain-containing Protein Ligand-of-Numb Protein-X (LNX)*

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Kerstin Sollerbrant‡, Elisabeth Raschperger¶, Momina Mirza¶, Ulla Engström, Lennart Philipson**, Per O. Ljungdahl‡, and Ralf F. Pettersson‡

From the Ludwig Institute for Cancer Research, Stockholm Branch, Karolinska Institutet, SE-17177 Stockholm, Sweden, the Ludwig Institute for Cancer Research, Uppsala Branch, SE-75124 Uppsala, Sweden, and the **Department of Cell and Molecular Biology, Karolinska Institutet, SE-17177 Stockholm, Sweden

The Coxsackievirus and adenovirus receptor (CAR) functions as a virus receptor, but its primary biological function is unknown. A yeast two-hybrid screen was used to identify Ligand-of-Numb protein-X (LNX) as a binding partner to the intracellular tail of CAR. LNX harbors several protein-protein interacting domains, including four PDZ domains, and was previously shown to bind to and regulate the expression level of the cell fate determinant Numb. CAR was able to bind LNX both in vivo and in vitro. Efficient binding to LNX required not only the consensus PDZ domain binding motif in the C terminus of CAR but also upstream sequences. The CAR binding region in LNX was mapped to the second PDZ domain. CAR and LNX were also shown to colocalize in vivo in mammalian cells. We speculate that CAR and LNX are part of a larger protein complex that might have important functions at discrete subcellular localizations in the cell.

For more than a decade, adenovirus has been used as vector to transfer genes of interest into several tissues in both animals and humans. It was long thought that adenovirus-based vectors could transfer genes promiscuously into almost all tissues and cell types. This optimistic view has, however, been revised; several obstacles for efficient gene transfer, including inaccessibility or complete lack of the primary virus receptor on certain cell types, have been identified. A more detailed investigation regarding the presence, regulation, and normal cellular function of the virus receptor in different tissues is therefore a prerequisite for efficient gene transfers.

The primary virus receptor was cloned in 1997 (1, 2). The same receptor is shared between two distinct virus groups, Coxsackie B and adenoviruses, and it has therefore been named CVADR (Coxsackie Virus and Adenovirus Receptor) or CAR.1 CAR is a member of the CTX subfamily of the large Ig superfamily characterized by one V-type and one C-type immunoglobulin domain in the extracellular domain, one membrane-spanning region, and an intracellular tail of varying length (for a review on CAR, see Ref. 3). Because of differential splicing, CAR is expressed as at least two isoforms, here termed CAR-1 and CAR-2 (4). The two isoforms differ only in the extreme C terminus located in the intracellular tail of the protein.

The cellular function for CAR is unknown, and no CAR-specific ligand has yet been identified. CAR might serve as a cell adhesion molecule and has been shown to mediate homophilic interaction with CAR molecules on neighboring cells (5, 6). One report suggests that CAR might function as a tumor suppressor, because the level of CAR expression inversely correlated with tumorigenicity (7). In polarized epithelial cells, CAR is located at the basolateral surface and specifically concentrates at tight junctions where it colocalizes with the PDZ domain protein ZO-1 (6). A large variation in CAR expression between tissues has been reported (1, 8, 9). CAR also seems to be developmentally regulated. Although high levels of CAR are expressed in the developing brain, expression is very limited in the adult (5, 8). CAR expression is also down-regulated in skeletal muscle during maturation and in oropharyngeal epithelium during differentiation (10, 11). Conditions such as differentiation of erythroid and myeloid cells, increased cell density of endothelial cells, and inflammation in heart muscle involve signals that up-regulate CAR levels in the adult, indicating that CAR expression is tightly regulated (12–14).

As a step toward understanding the normal function of CAR, we have searched for proteins interacting with the intracellular tail of CAR. Both CAR-1 and CAR-2 harbor in their respective C termini possible binding sites for PDZ domain-containing proteins (4, 15). We used the complete CAR-1 intracellular tail from mouse in a yeast two-hybrid screen and identified the Ligand-of-Numb protein-X (LNX) as an interacting partner with CAR. LNX is a multi-PDZ-containing protein previously identified as a binding partner for Numb, a protein implicated in asymmetric cell division and regulation of Notch activity in the developing nervous system in Drosophila (16, 17). We also analyzed the regions in CAR and LNX involved in the interaction, and we demonstrated colocalization of CAR and LNX in mammalian cells.

EXPERIMENTAL PROCEDURES

DNA Constructs

Yeast Expression Plasmids—pGBT9 and pACT-2 were purchased from Clontech. pGal4DBD—mCARic (DBD/CAR) (bait) harbors the GAL4 DNA binding domain fused to the complete intracellular tail of mouse CAR-1 and was constructed by PCR amplification of pBKCM-VmCAR using primers 104 and 103. The resulting fragment was di-
gusted with EcoRI and cloned into EcoRI/SmaI in pGBT9. pHA/tLNX is the original mouse cDNA rescued in the yeast two-hybrid screen and contains a truncated LNX cDNA (nucleotides 334–2144 in LNXp70, accession number AF034746) in pACT-2. pHA/Wt p80 contains a double HA-tag and was constructed by transfer of an HA-Wt p80 fragment from the corresponding mammalian expression vector by EcoRI/Klenow/XhoI treatment into pACT-2 vector treated likewise. pHA/Wt p70 was constructed by transfer of a Wt p70 fragment from the mammalian expression vector pWt p70 treated with EcoRI/mungbean nuclelease/XhoI into pACT-2 treated likewise. pEXR is a PACT-2-derived plasmid expressing the activation domain of the transcription factor RXX.

**Mammalian Expression Plasmids—pHAANX, pHAANX-PDZ (1, 2), pHAANX-PDZ (1)** were constructed by standard PCR amplification using yeast pHAANX as template. A common 5′-primer, No. 88, was used with 3′-primers, Nos. 89, 92, and 91, respectively. PCR fragments were digested with XhoI and cloned into pBKCVMV vector (Stratagene) treated with EcoRI/Klenow/XhoI, pHAANX-PDZ (3, 4) was constructed by treating pHAANX with BspHI/Klenow/XhoI and inserting a 0.7-kb fragment into the vector part of pHAANX-PDZ (1) treated with BsaI/mungbean nuclelease/XhoI. pHAANX-PDZ (2) was constructed by self-ligation of pHAANX-PDZ (1, 2) treated with BsaI/mungbean nuclelease/PmlI. pHA/Wt p80 was constructed by nested RT-PCR on RNA prepared from mouse lung. RT reaction was performed using the SuperScript First Strand System (Invitrogen) according to the manufacturer's directions. Primer pairs were Nos. 141 and 142 and Nos. 123 and 91. The resulting 1.1-kb fragment was digested with EcoRI/PmlI and ligated into pHAANX treated likewise. pHA/Wt p70 was cloned by excision of Wt p70 from pWt p70 by EcoRI/XhoI/mungbean nuclelease treatment and ligation downstream of an HA-tag in a pdCA3.1-derived vector plasmid. pHA/Wt p80 was constructed by RT-PCR using RNA prepared from mouse lung as template. RT reaction was performed using the SuperScript First Strand System according to the manufacturer’s directions. PCR primers were Nos. 141 and 89. The resulting 2.3-kb fragment was digested with XhoI and ligated to pdCA3.1(zeoc +) vector (Invitrogen) digested with EcoRI/XhoI. To construct pWt p70, RT-nested PCR was performed using total RNA from mouse lung with the following primer pairs, A, Nos. 17 and 16; B, Nos. 122 and 91. The amplified fragment was digested with EcoRI/PmlI and cloned into pHAANX digested likewise. pBKCVMVcar expresses the entire mouse CAR-1 cDNA and has been described as pRTMR (1).

**GST Plasmids—pGST** was purchased from Amersham Biosciences (pGEX-2T), pGST-CAR, pGST-CAR-TTV, and pGST-CAR-RC terms were constructed by standard PCR amplification using pBKCVMVcar as template, a common 5′-primer, No. 125, and 3′-primers Nos. 126, 130, or 129, respectively. The PCR fragments were digested with EcoRI/SmaI and cloned into pGEX-2T in the corresponding sites. The sequence of all PCR primers can be found at www.lcr.ki.se. The relevant portions of all new plasmid constructs were sequenced to verify correct DNA sequences.

**Yeast Two-hybrid and cDNA Library Screening**

The screen, transformation, and selection procedures were all performed according to instructions by the manufacturer (Clontech, Matchmaker GAL4 two-hybrid system). Briefly, a pACT2 vector-based yeast strain 17-day mouse embryonic cDNA library was purchased from Clontech. The bait pGAL4DBD—mCARic construct and the library were transformed sequentially into the yeast strain AH109 (Clontech), and the transformants plated on medium lacking amino acid selections. Individual colonies lacking amino acids His, Leu, and Trp. In total, 3 × 10⁶ clones were screened. Emerging colonies were replica-plated on high-stringency selection plates lacking amino acids Ase, His, and Trp. A colony lift assay to test β-galactosidase expression was then performed. Rescue of library-derived plasmids was done in the bacterial strain KCC8 (Clontech) that has a defect in leuB that can be complemented by yeast U2.

**Mammalian Cell Lines, Culture Conditions, and Transfection**

293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin (all from Invitrogen). Cells were cultured at 37 °C in a CO₂-humidified incubator. Lipofectamine 2000 reagent (Invitrogen) was used for plasmid transfection of cells using conditions that were recommended by the manufacturer.

**Expression of GST Fusion Proteins**

GST fusion constructs were expressed in Escherichia coli BL21 competent cells using conditions recommended by the manufacturer (Amersham Biosciences). Equal amounts of purified GST fusion proteins were separated on a 10% SDS-PAGE gel under reducing conditions and visualized by staining with 0.25% Comassie Brilliant Blue (BioRad).

**Preparation of Mammalian Protein Extract, GST Pull-down Assay, and Western Blot**

293 cells were transfected with 10 μg of LNX plasmid in 10-cm petri dishes. 24 h later, proteins were extracted by incubating cells in 500 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1% Triton X-100, EDTA-free 1× complete protease inhibitor mixture tablets (Roche) on ice for 15 min. Lysates were centrifuged for 10 min at maximum speed in an Eppendorf centrifuge at 4 °C. 250 μl of supernatant was then mixed with 250 μl of lysis buffer and incubated with GST fusion protein in the presence of glutathione-Sepharose 4B at 4 °C for 90 min. After several washes in lysis buffer, bound proteins were separated on a 10% SDS-PAGE gel under reducing conditions and transferred to Protran nitrocellulose transfer membrane (Schleicher & Schuell). Membrane was incubated in blocking buffer (standard phosphate-buffered saline with 0.1% Tween 20 (1× phosphate-buffered saline-T) and 5% dry milk) overnight at 4 °C, treated with an HA-specific polyclonal antibody (Clontech) diluted 1:100 in blocking buffer at room temperature for 1 h, washed in 1× phosphate-buffered saline-T, incubated with HRP-labeled anti-ribbon IgG (Amersham Biosciences) diluted 1:4000 in blocking buffer at room temperature for 1 h, and washed again. Peroxidase activity was detected using enhanced chemiluminescence and Hyperfilm ECL (Amersham Biosciences).

**In Vitro Transcription/Translation**

The TNT T7 quick-coupled transcription/translation system (Promega) was used according to instructions. [35S]Met-labeled Wt p80 protein was prepared by adding mammalian pWt p80 directly to the reaction mix. To prepare labeled Wt p70, PCR amplification using mammalian pWt p70 as template was first done to introduce a T7 promoter. Primers were Nos. 131 and 89. For a description of the primers, see www.lcr.ki.se. Labeled protein extract was diluted to 1 ml in lysis buffer (see above), and 500 μl was used in GST pull-down assays performed as described above.

**Production of a Polyclonal Antiserum toward CAR-1**

A peptide encompassing the last 13 amino acids of human CAR-1 (FKYPYKTDGITVV) was coupled to mouse maleimide activated mcKLH carrier (Pierce) via a Cys residue added to the N terminus and used to immunize rabbits according to standard protocols. The resulting antiserum (RP194) cross-reacts with the mouse CAR-1 homologue.

**Indirect Immunofluorescence**

293 cells grown on 6-cm petri dishes were transfected with 0.25 μg of pBCKMVMcar and/or 1.7 μg of LNX construct. Six hours post-transfection, cells were split and transferred to coverslips in 6-well plates. At 24 h post-transfection, indirect immunofluorescence on formaldehyde-fixed cells was performed as described in Ref. 18 except that the antibody incubation time was increased to 1 h. Primary antibodies were rabbit polyclonal CAR-1-specific RP194 (see above) and monoclonal clone HA-7 (Sigma), both diluted 1:500. Secondary antibodies were fluorescein isothiocyanate conjugated goat anti-rabbit IgG (Sigma) and Alexa Fluor 546 goat anti-mouse IgG (Molecular Probes), both diluted 1:200. Stained proteins were analyzed in a Zeiss Axiosplan fluorescence microscope.

**RESULTS**

**Identification of a Protein Interacting with the Cytoplasmic Tail of CAR—To identify proteins interacting with the intracellular tail of mouse CAR-1 (Fig. 1C), a yeast two-hybrid screen was performed. The complete intracellular tail of CAR-1 was expressed as a fusion protein with the DNA binding domain (DBD) of the yeast transcription factor GAL4 by cloning into the yeast expression vector pGBKT9. In the resulting bait plasmid (DBD/CAR), GAL4 DBD is located in the 5′-end to maintain a free C terminus of CAR-1 in the expressed fusion protein. DBD/CAR was cotransformed with a 17-day mouse embryo cDNA library in the yeast strain AH109. Proteins expressed from the pACT-2-derived library all contained an HA-
tag in the N terminus. Of the $3 \times 10^6$ clones screened, 31 cDNAs were identified as positive based on growth on selective media and activation of the lacZ reporter. Library-derived plasmids were rescued and retransformed together with bait plasmid in AH109 to verify the phenotype. Yeast transformed with cDNA only that was able to grow on high-stringency selection plates, as well as yeast transformed with cDNAs that expressed proteins that bound to the Gal4 DNA binding domain, were eliminated from the screen. Sequencing of the four remaining cDNAs identified two of the clones as LNX and the remaining two clones as the closely related protein LNX2. In this report we have focused on LNX. None of the four clones was full-length cDNA. The N-terminal truncated LNX was therefore called HA/tLNX (Fig. 1A). Fig. 2A, right panel shows that the interaction between HA/LNX and CAR was specific because growth of blue colonies on high-stringency-selective plates containing X-a-Gal was only apparent in the presence of DBD/CAR. Yeast transformed with HA/LNX and DBD alone or with HA/LNX only were not able to grow. Transformation per se did not affect growth of the yeast host because all three types of transformants were capable of growing on rich, nonselective yeast peptone dextrose (YPD) medium (Fig. 2A, left panel) as well as on plates selecting for the presence of bait and/or cDNA plasmids (data not shown).

Wild-type LNX is expressed as two isoforms, Wt p70 and Wt p80, that differ only in the N terminus (Fig. 1A). Both Wt p70 and p80 harbor four PDZ domains and an LDNPAY sequence that previously was shown to be important for binding to the Numb protein (16). In addition, Wt p80 harbors a RING finger domain. All of these conserved protein domains have in other proteins been shown to mediate protein-protein interactions. To see whether full-length LNX could interact with CAR, Wt p80 and p70 were cloned in pACT-2 and transformed in yeast as was done for HA/LNX. The resulting transformants were tested for growth on selective and nonselective plates (Fig. 2B, C and D, respectively). Both LNX isoforms showed the same phenotype as HA/LNX. As an additional control, we performed the same experiment using a cDNA encoding the activation domain of the transcription factor RXR (Fig. 2D). No growth on selective media was seen when cotransformed with DBD/CAR, indicating that CAR does not exhibit unspecific binding to any protein containing a transcription activation domain. Together these experiments showed that mouse HA/Wt p70, HA/Wt p80, and HA/LNX all specifically bind to the intracellular tail of mouse CAR-1 in vivo in yeast.

**LNX PDZ Domain No. 2 Interacts with the Intracellular Tail of CAR in Mammalian Cell Extracts**—The yeast experiments suggested that the domain of LNX responsible for binding to CAR was located in one or more of the PDZ domains because HA/Wt p70, HA/Wt p80, as well as HA/tLNX bound CAR. To

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**Fig. 1. Schematic representation of DNA constructs and the unique C-terminal sequences of CAR-1 and CAR-2.** A, mouse LNX constructs with color-coded protein domains. Blue boxes represent PDZ domains 1–4. The RING finger domain is shown in yellow, the HA-tag in red, and the LDNPAY sequence is marked as a black line. B, GST fusion constructs. GST/CAR expresses a fusion protein between GST and the complete intracellular tail of mouse CAR-1. GST/CAR7TVV and GST/CAR4C-term are C-terminal deletions of GST/CAR. Numbers indicate the amino acids of CAR present in the construct. C, amino acid sequence of the two different C termini of CAR-1 and CAR-2. Gray box represents the part of CAR common to both isoforms.

**Fig. 2. Growth of yeast transformants on YPD and high-stringency selection plates.** One colony of each of the indicated yeast transformants was resuspended in 0.5 ml of water and streaked onto rich yeast peptone dextrose (YPD) medium (left panel) or high-stringency selection plates containing X-a-Gal (right panel). Yeast cells were transformed with the following plasmids: pHA/LNX alone (tLNX), pHA/LNX and pGBT9 (tLNX+DBD), or pHA/LNX and pGal4DBD-mCARic ([LNX]+DBD/CAR) (A); pHA/Wt p70 alone (p70), pHA/Wt p80 and pGBT9 (p80+DBD), or pHA/Wt p80 and pGal4DBD-mCARic (p80+DBD/CAR) (B); pHA/Wt p70 alone (p70), pHA/Wt p70 and pGBT9 (p70+DBD), or pHA/Wt p70 and pGal4DBD-mCARic (p70+DBD/CAR) (C); pRXR alone (RXR), pRXR and pGBT9 (RXR+DBD), or pRXR and pGal4DBD-mCARic (RXR+DBD/CAR) (D).
verify the interaction in mammalian cells and to map the interacting domain in LNX, HA/Wt p70 as well as HA/tLNX and PDZ deletions thereof were recloned together with the pACT-2-derived N-terminal HA-tag in a mammalian expression vector (Fig. 1A). The different HA-tagged LNX constructs were transfected into 293 cells. Protein extracts were prepared and used for in vitro binding to GST or to a GST-CAR fusion protein (Fig. 1B). Bound proteins were isolated, separated on SDS protein gels, transferred to filters, and visualized by Western analysis using an HA-specific antibody (Fig. 3). Unbound protein extract (input) was included as a control for transfection efficiency and to show that proteins of expected size were expressed. The HA-antibody recognized a 70-kDa protein in untransfected 293 cell extract (lane 1). This nonspecific band, however, disappeared following binding to the GST proteins (lanes 2 and 3). Only HA/LNX-specific signals therefore remained in the binding experiments when transfected cell extracts were used.

HA/LNX bound to GST-CAR, thus verifying the interaction seen in the yeast system (Fig. 3, lane 9). Constructs harboring either PDZ 1 and 2 (HA/tLNX-PDZ (1, 2), lane 15) or PDZ 2 alone (HA/tLNX-PDZ (2), lane 18) retained binding to GST-CAR. In contrast, constructs harboring only PDZ 3 and 4 (HA/tLNX-PDZ (3, 4), lane 12) or PDZ 1 alone (HA/tLNX-PDZ (1), lane 21) did not bind to GST-CAR. To verify binding to full-length LNX, HA/Wt p70 were tested and also found to bind GST-CAR (lane 6). No binding to GST alone was seen with any of the HA/LNX constructs (lanes 5, 8, 11, 14, 17, 20). Similar amounts of GST fusion proteins were used in all binding reactions (Fig. 4B, upper bands). Together these results confirmed the interaction between LNX and CAR in mammalian cell extracts and also identified PDZ domain 2 as being important for binding to the intracellular tail of CAR.

Sequences in CAR-1 Involved in Binding to LNX—We next wanted to identify the region in CAR involved in the interaction with LNX, and GST-CAR was therefore truncated at two positions (Fig. 1B). PDZ domains of the class I type have previously been shown to interact mainly with the extreme C-terminus of target proteins that ends with the consensus sequence T/S-X-bulky amino acid, where X is any amino acid (15). Consistent with this observation, the binding seen between GST-CAR and HA/LNX was almost completely abolished when the binding was instead carried out with GST-CAR TVV, indicating that the last three amino acids of CAR are indeed important for binding (Fig. 4A, compare lanes 2 and 3). Surprisingly, GST-CAR A Cterm, which expresses a fusion protein lacking the last 13 amino acids of CAR, could still bind HA/tLNX despite the absence of the C-terminal TVV residues (Fig. 4A, lane 4). This result suggested that other sequences besides the TVV terminus are involved in binding to LNX. The fact that an affinity column harboring a peptide encompassing the last 13 amino acids of CAR-1 was not able to retain HA/tLNX from cell extracts further argued for this conclusion (data not shown). In GST-CAR A Cterm the part of the CAR intracellular tail expressed is identical in both splice variants of CAR, suggesting that LNX might also bind to CAR-2 (Fig. 1C). Indeed, a fusion construct between GST and the complete intracellular tail of the human CAR-2 homologue interacted with HA/tLNX in a GST pull-down assay (data not shown). The interaction between CAR and HA/tLNX was specific, because no binding to GST alone was seen (Fig. 4A, lane 1). All GST fusion proteins were used in equal amounts (Fig. 4B, upper bands). Together

![Fig. 3. Analysis of the region in LNX required for binding to CAR.](image)

![Fig. 4. Analysis of the region in CAR required for binding to LNX.](image)
these experiments indicated that binding to LNX may require both an intact TVV C terminus in mCAR-1 and a region of CAR present in both CAR splice variants.

Wild-type, Untagged LNX p70 and LNX p80 Bind to the Intracellular Tail of CAR—The in vivo binding experiments carried out in yeast as well as the in vitro binding experiments using mammalian cell extracts were all done with LNX proteins harboring an HA-tag in the N terminus. To investigate whether untagged LNX proteins could interact with CAR, both full-length LNX proteins were expressed from a T7 promoter in rabbit reticulocyte lysate in the presence of [35S]Met. Labeled proteins were used in a GST pull-down assay, separated on an SDS protein gel, and visualized by autoradiography (Fig. 5, A and B). As can be seen in the input control lanes, only one band of the expected size was synthesized (lane 1). No labeled band was seen in the absence of DNA template in the coupled in vitro transcription/translation reaction (data not shown). Both Wt p70 and Wt p80 bound to GST-CAR with comparable efficiencies (lane 3). The binding was specific because no binding was seen to GST alone (lane 2). This experiment showed that both isoforms of untagged full-length LNX proteins were able to specifically bind to CAR. Both in vitro-synthesized full-length LNX proteins showed considerable less binding to GST-CARΔTVV compared with GST-CAR (compare lanes 3 and 4). Binding was retained when binding was instead done with GST-CARΔCterm (lane 5). These results were in agreement with the binding obtained using extract from HA/tLNX-transfected cells (Fig. 4A), thus supporting the conclusion that there might be more than one region in CAR-1 involved in the interaction with LNX.

Full-length LNXp70, LNXp80, and tLNX Colocalize with CAR in 293 Cells—CAR has previously been reported to localize to the plasma membrane and to accumulate at cell-cell contacts (6). To investigate whether LNX and CAR colocalized in vivo, 293 cells were cotransfected with plasmids expressing CAR-1 and HA-tagged LNX. The proteins were localized by indirect immunofluorescence using CAR-1- and HA-specific antibodies, respectively (Fig. 6). HA/tLNX as well as HA/Wt p70 and HA/Wt p80 were all found to colocalize with CAR at cell-cell contacts, although considerable staining of all three LNX proteins was also seen in the cytoplasm and in the nucleus (Fig. 6, A, B, and C, respectively). Cells transfected with the LNX constructs alone did not localize to cell-cell contacts, indicating that the amount of endogenous CAR present in these cells was not enough to visualize colocalization between CAR and LNX.

Fig. 5. Binding of untagged full-length LNXp70 and LNXp80 to CAR. Wt p70 (A) and Wt p80 (B) were translated in vitro in the presence of [35S]Met and incubated with the indicated GST fusions. Bound proteins were separated on a 10% SDS-PAGE gel. The gel was dried, and labeled proteins were analyzed by autoradiography. Input: 10% of the labeled extract used in the binding reactions.

Fig. 6. Colocalization of CAR-1 and LNX in 293 cells. pBRCMVmCAR was cotransfected with pHAtLNX (A), pHAt Wt p70 (B), or pHAt Wt p80 (C) in 293 cells. 24 h post-transfection, CAR-1 and LNX were localized by indirect double immunofluorescence. CAR-1 was detected with a CAR-1-specific polyclonal antibody and a fluorescein isothiocyanate-conjugated anti-rabbit secondary antibody. HA-tagged proteins were detected with an HA-specific monoclonal antibody and Alexa Flour 546 anti-mouse secondary antibody. Right panels show merged images.
(data not shown). No bleed-through between channels was observed. Together these results showed that CAR is able to recruit LNX to cell-cell contact sites of the plasma membrane.

**DISCUSSION**

In this study we have identified LNX as a protein that binds to the intracellular tail of CAR-1. The interaction between CAR and LNX was shown both in *vivo* in yeast and *in vitro*, and the proteins were also shown to colocalize in mammalian cells. Binding did not appear to be confined to the conserved PDZ motif-binding region in the extreme C terminus of CAR-1 but was also found in upstream sequences common to both CAR splice variants. Although binding to C-terminal sequences appears to be the typical mode of interaction, PDZ domains can also interact with internal amino acid sequences within ligands (19). Why a deletion of the last 13 amino acids of CAR-1 retained binding to LNX whereas a truncation of the last three did not is at present unclear but might be due to masking of the upstream binding site because of conformational changes in the CAR\_7TV protein.

LNX was originally isolated as a binding partner to Numb, a protein implicated in the control of cell fate decisions during development in Drosophila (16, 17). Numb antagonizes the Notch signaling pathway, a process that is regulated through asymmetric cell division in which Numb and Notch segregate to different daughter cells (20–22). Numb and Notch are both evolutionarily conserved proteins. Homologues of the proteins responsible for asymmetric localization of Numb in Drosophila have not been found in the mammalian system, and the mechanism for this process in mammals therefore remains unknown. Efficient binding between LNX and Numb requires the NPAY sequence motif as well as the first PDZ domain in LNX. We demonstrate that CAR interacts with the second PDZ domain of LNX. Because CAR and Numb interact with different domains of LNX, the possibility exists that these three proteins are present in the same multi-protein complex. This raises the possibility that one function of CAR is to regulate the localization of LNX and Numb to specific sites at the plasma membrane. It is interesting to note that LNX2 interacts with Numb and that expression of Numb and LNX/LNX2 were found to overlap in the developing and adult mouse brain (23). The fact that CAR also interacts with both LNX family members and that CAR is expressed at high levels in the developing mouse brain further strengthens the idea that CAR, LNX, and Numb might form a functional complex. We have so far been unsuccessful in co-precipitating endogenous CAR and LNX. More work is thus needed to unequivocally demonstrate such an interaction in cultured cells or tissues.

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