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Coxsackievirus and adenovirus receptor (CAR) mediates trafficking of acid sensing ion channel 3 (ASIC3) via PSD-95

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ABSTRACT

We have previously shown that the Coxsackievirus and adenovirus receptor (CAR) can interact with post-synaptic density 95 (PSD-95) and localize PSD-95 to cell–cell junctions. We have also shown that activity of the acid sensing ion channel (ASIC3), a H^+-gated cation channel that plays a role in mechanosensation and pain signaling, is negatively modulated by PSD-95 through a PDZ-based interaction. We asked whether CAR and ASIC3 simultaneously interact with PSD-95, and if so, whether co-expression of these proteins alters their cellular distribution and localization. Results indicate that CAR and ASIC3 co-immunoprecipitate only when co-expressed with PSD-95. CAR also brings both PSD-95 and ASIC3 to the junctions of heterologous cells. Moreover, CAR rescues PSD-95-mediated inhibition of ASIC3 currents. These data suggest that, in addition to activity as a viral receptor and adhesion molecule, CAR can play a role in trafficking proteins, including ion channels, in a PDZ-based scaffolding complex.

1. Introduction

The Coxsackievirus and adenovirus receptor (CAR) is both a cell adhesion protein and a viral receptor [1,2]. CAR is a developmentally essential member of the immunoglobulin superfamily and is present in numerous cell types, including many regions of the nervous system where it can play a role in endocytosis and cargo trafficking in neurons [3–8]. CAR contains a class 1 PSD-95/*Drosophila* discs-large protein/zonula occludens protein-1 (PDZ)-binding domain at its C-terminus and is known to interact with and affect the trafficking of several PDZ domain-containing scaffolding proteins, such as PSD-95, MAGI-1, PICK1, and MUPP-1 [2,9].

The PDZ domain-containing family is an important group of proteins involved in the transport and stabilization of channel complexes and adhesion molecules [10,11]. These are generally large scaffolding proteins containing one or more PDZ domains, along with several other protein domains. One key PDZ domain-containing protein involved in the localization and stability of many ion channels is post-synaptic density 95 (PSD-95). PSD-95 contains three PDZ domains, an SH3 domain, and a guanylate kinase domain, and each of these domains potentially interacts with several different partners. PSD-95 also interacts with itself increasing the potential number of simultaneous interactions. For example, PSD-95 is known to concentrate multiple different ion channels and other synaptic proteins at glutamatergic synapses [10]. However, it is unclear whether PSD-95 functions to traffic channels to the synapse, or whether it serves as a scaffold able to trap and retain channels there after arrival [12]. We have previously shown that CAR is able to direct PSD-95 localization to the junctions of heterologous cells [2], suggesting that CAR might also participate in the localization of other proteins, including cell surface signaling proteins, within a larger protein complex.

Acid sensing ion channels (ASICs) are proton-gated cation channels with four alternatively spliced members (ASIC1a, 1b, 1b2, 2a, 2b, 3, and 4) [13,14]. ASIC channels function as homo- or hetero-multimers, and they interact with multiple other modulatory proteins, including PDZ domain-containing proteins. ASIC channels are known to be involved in nociception and in fear response, and may be important for other pathogenic or psychiatric diseases. In particular, ASIC3 expression is primarily restricted to peripheral sensory neurons where it plays a role in sensing pain associated with modest drops in pH and may play a role in mechanosensation. PSD-95 is also implicated in pain pathways [15]. ASIC3 directly

Abbreviations: CAR, Coxsackievirus and adenovirus receptor; ASIC3, acid sensing ion channel 3; PSD-95, post-synaptic density 95; PDZ, PSD-95/*Drosophila* discs-large protein/zonula occludens protein; Ab, antibody; IP, immunoprecipitation.

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interacts with PSD-95 via a PDZ–PDZ binding domain interaction [16,17]. Interestingly, this interaction increases retention of ASIC3 within the reticular compartments of the cell where it strongly co-localizes with PSD-95. This interaction reduces ASIC3 cell surface levels and hence proton-gated current.

We asked whether CAR and ASIC3 could simultaneously interact with PSD-95, and if so, what the formation of this complex does to the localization and activity of the individual proteins. We show that CAR is able to bring both PSD-95 and ASIC3 to the junctions of heterologous cells resulting in restoration of ASIC3 current, as measured by whole-cell patch-clamp. These results suggest a novel function of CAR as a trafficking protein for cell surface signaling molecules.

2. Materials and methods

2.1. Materials

FLAG M2 antibody (Ab) was purchased from Sigma (F3165, St. Louis, MO). Guinea pig ASIC3 Ab was purchased from Millipore (AB5927, Billerica, MA). Mouse anti-HA was purchased from Cell Signaling Technology (2367S, Danvers, MA). HRP conjugated donkey anti-guinea pig Ab was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Alexa-488, -568, or -647 conjugated goat anti-mouse, -rabbit, or -guinea pig Abs, mouse and rabbit anti-GFP were from Molecular Probes (Eugene, OR). Mouse anti-CAR RmcB Ab (CRL-2379, ATCC, Manassas, VA) was produced by the University of Iowa Hybridoma Core. Rabbit anti-CAR 1605p was produced in rabbits immunized with a GST fusion to the intracellular CAR C-terminus (aa 261–365) as previously described [18]. COS-7 cells were from ATCC (Manassas, VA), and maintained under standard culture conditions (D-MEM with 10% FCS, penicillin and streptomycin).

2.2. Transfection

COS-7 cells were electroporated by standard methodologies [2]. Briefly, 10 million cells were mixed with 10 μg of each DNA for triple transfections, in 400 μl of cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO4, 10 mM KH_2PO4, 25 mM Hepes, 2 mM EGTA, 5 mM $MgCl_2$, 2 mM ATP, and glutathione) and incubated in an electroporation cuvette (Bio-Rad Laboratories, Hercules, CA) for 30 min on ice. For double or single transfections, 10 μg of the target plasmid DNA was balanced by either peGFP (Clontech) or parental pcDNA3.1. After electroporation, cells were seeded onto 10 cm dishes for immunoprecipitation (IP), collagen coated glass chamber slides for immunofluorescence, or glass coverslips for patch-clamp studies 2 days later.

2.3. Immunostaining

COS-7 cells grown on collagen coated chamber slides were washed once with PBS, fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 2% BSA in SuperBlock (Pierce, Rockford, IL). Cells were incubated with primary Ab, washed extensively and incubated with Alexa-labeled secondary Ab. After washing, slides were coverslipped with Vectashield mounting media (Vector Laboratories, Burlingame, CA). Images were acquired with an FV1000 Laser Scanning Confocal Microscope (Olympus, CenterValley, PA) using a 60× oil immersion lens.

2.4. IP and Western blot

COS-7 cells from two 100 mm plates were placed on ice, washed once with ice cold PBS, and lysed with lysis buffer [50 mM Tris–HCl, pH 7.5, 137 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM EGTA, protease inhibitors (10 μg/ml) leupeptin, aprotinin, pepstatin, and 1 mM phenylmethylsulfonyl fluoride] by rocking at 4 °C. Cells were scraped, sonicated five times and spun in a microcentrifuge at full speed for 10 min. For co-IP, supernatant was incubated with the indicated Ab with rotation at 4 °C overnight. Protein A or G conjugated Sepharose (Amersham Biosciences, Uppsala Sweden) was added for 1–2 h followed by a wash with lysis buffer, 10% lysis buffer in TBS [50 mM Tris–HCl, pH 7.5, 137 mM NaCl], and TBS. Beads were suspended in loading buffer [4% sodium dodecyl sulfate, 100 mM dithiothreitol, 20% Glycerol, 65 mM Tris, pH 6.8, 0.005% bromophenol blue] and proteins were separated by SDS–polyacrylamide gel electrophoresis. Gels were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), blocked with 5% BSA, washed, probed with primary Ab as indicated, followed by washing and incubation with HRP-conjugated donkey anti-mouse or -rabbit secondary Ab (Jackson ImmunoResearch Laboratories, West Grove, PA). Bands were detected with ECL reagents (Pierce, Rockford, IL) and imaged on the EpiChemi^3 Darkroom (UVP Inc., Upland, CA).

2.5. Electrophysiology

Whole-cell patch-clamp recordings (at −70 mV) from COS-7 cells were performed at room temperature with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) and were acquired and analyzed with PULSE/PULSEFIT 8.70 (HEKA Electronics, Lambrecht, Germany) and IGOR Pro 6.01 (WaveMetrics, Lake Oswego, OR) software. Currents were filtered at 5 kHz and sampled at 2 kHz. Series resistance was compensated by at least 50%. Capacitive currents were compensated for and recorded for normalization of peak current amplitudes (reported as current densities). Micropipettes (2–4 MΩ) were filled with internal solution (mM): 100 KCl, 10 EGTA, 40 Hepes, and 5 $MgCl_2$, pH 7.4, with KOH. External solutions contained (mM): 120 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 Hepes, 10 MES; pH was adjusted with tetramethylammonium hydroxide, and osmolarity adjusted with tetramethylammonium chloride (TMA-Cl). Extracellular solutions were changed within 20 ms by using a computer-driven solenoid valve system [19]. Kinetics of desensitization was fit to with single exponential equations and time constants (τ) reported. Data are means ± SEM. Statistical significance was assessed using unpaired Student's *t*-test.

3. Results

3.1. ASIC3, CAR, and PSD-95 interact in a trimolecular complex

We have previously demonstrated interactions between ASIC3 and PSD-95, as well as CAR and PSD-95 [2,16]. Moreover, both interactions have been shown to be dependent on the functional PDZ-binding domains of ASIC3 and CAR. To investigate whether CAR interacts directly with ASIC3, COS-7 cells were co-transfected with paired combinations of plasmids encoding HA-tagged ASIC3, FLAG-tagged CAR, and GFP-tagged PSD-95 (Fig. 1A). As expected, coexpression of ASIC3 with PSD-95 allowed for co-immunoprecipitation of the two proteins. However, CAR, when coexpressed with ASIC3, did not co-immunoprecipitate with ASIC3 (Fig. 1A). In contrast, when all three proteins were coexpressed, ASIC3 co-immunoprecipitated both PSD-95 and CAR (Fig. 1B). Similarly, PSD-95 co-immunoprecipitated ASIC3 and CAR, and CAR co-immunoprecipitated ASIC3 and PSD-95 upon coexpression of all three proteins (Fig. 1C). We next investigated whether this trimolecular interaction was dependent upon PDZ-binding domain interactions. Deletion of the four terminal amino acids comprising the PDZ-binding domain of ASIC3 (ASIC3Δ4) abolished the capacity