The Coxsackie and adenovirus receptor (CAR) binds microtubules and plays a role in cell migration

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Running title: Interaction of CAR with microtubules

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Summary

The Coxsackie and adenovirus receptor (CAR), a cell adhesion molecule of the immunoglobulin superfamily, inhibits cell growth of a variety of tumors. The cytoplasmic domain of CAR has been implicated in decreased invasion and intracerebral growth of human U87 glioma cells. Using affinity binding, we identified tubulin as an interaction partner for the cytoplasmic domain of CAR. The interaction was specific; CAR and tubulin co-immunoprecipitated in cells expressing endogenous CAR and partially co-localized \textit{in situ}. The binding of CAR to tubulin heterodimers and to microtubules was direct, with dissociation constants of \~1 µM for tubulin and \~32nM for \textit{in vitro} assembled microtubules. While CAR expressing U87 glioma cells had decreased migration in a chemotactic assay in Boyden chambers as compared to control cells, an effect which depended on the presence of the cytoplasmic domain of CAR, the difference was abrogated at low, non-cytotoxic doses of the taxane paclitaxel, a microtubule-stabilizing agent. These results indicate that CAR may affect cell migration through its interaction with microtubules.
also been shown to mediate transendothelial migration of neutrophils (16).

The V-type Ig domain of CAR is sufficient for adenovirus binding (17,18) and the transmembrane and cytoplasmic domains of CAR are not required in adenovirus infection (19). Nevertheless, the cytoplasmic domain of CAR has several features of potential functional importance for the molecule. The cytoplasmic domain of CAR is highly conserved between species and constitutes nearly one third of the entire molecule. The membrane-proximal cysteines 259 and 260 provide a palmitoylation motif required for stable plasma membrane expression of CAR (20). Several regions are involved in basolateral targeting in epithelial cells (21). Furthermore, both full-length isoforms of CAR contain PDZ domain binding sequences; these isoforms are identical over much of their length, including the transmembrane and most of the cytoplasmic domain, but, through alternative splicing, differ at their C-termini [CAR-SIV versus CAR-TVV]. Accordingly CAR has been shown to bind the PDZ domain-containing proteins Ligand-of-Numb Protein-X (LNX) (22,23), MAGI-1b and PICK1 (24), ZO-1 (13) and MUPP-1 (25).

Whereas their normal tissue counterparts express readily-detectable levels of CAR, low or absent expression of CAR is seen in many primary tumor tissue and cell lines (26-29). Forced expression of CAR results in inhibition of tumor cell growth in human prostate cancer (30), bladder cancer (31), and glioma (32) cell lines. We have recently shown that rates of glioma cell invasion and tumor growth are reduced by forced expression of full-length CAR but not by expression of cytoplasmic domain-deleted CAR, indicating the C-terminal cytoplasmic domain of CAR is required for inhibition of glioma cell invasion and tumor growth (33).

Fusion Proteins
To create GST-CAR fusion protein the intracellular domain (nucleotides 778-1098, encoding amino acids 259-365) of CAR (GenBank Accession Y07593) was cloned into pGEX-3X plasmid (Amersham Biosciences, Piscataway, NJ, USA). BL21 bacteria (Promega, Madison, WI, USA) were transformed with pGEX-3X-CAR (778-1098). GST and GST-CAR fusion proteins were produced by overnight growth, followed by 4 hour induction with 1mM and 0.5mM of IPTG (Fisher Scientific, Ottawa, ON, Canada), respectively. Bacterial cells were sonicated in PBS with protease inhibitor cocktail (Roche, Mississauga, ON, Canada). Cleared bacterial lysate was incubated with glutathione-Sepharose beads (Amersham Biosciences) for 5 min at room temperature. 

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture
U87CAR and U87LNCX cell lines have been described and characterized previously (33). Briefly, they were generated from the parental human glioma U87-MG cell line by infection with a retroviral vector expressing either full-length CAR [m1 isoform (34)] or an empty retroviral vector, respectively. They both consist of pooled populations of clones. The U87CAR cells express CAR at levels equivalent to that observed in lysates prepared from post-natal day 6 mouse brain (33). The U87CAR781 cell line has also been described and characterized previously (33). The pooled clones were generated through retroviral infection with a truncated CAR construct containing amino acids 1-260. The human bladder 253J cell line was obtained from Dr. Hsieh (MD Anderson, Houston, TX), and expresses robust levels of endogenous CAR (31). The additional CAR-expressing cell lines, human embryonic kidney 293A and human cervical HeLa cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained (unless stated otherwise) at 37°C with 5% CO₂ in Dulbecco's Modified Eagle Medium (D-MEM) supplemented with 2mM L-glutamine, 10% heat inactivated fetal bovine serum (FBS) and an antibiotic cocktail [final concentration of 30 µg/ml gentamicin, 100 units of penicillin/ml, and 100 µg of streptomycin/ml] (Invitrogen, Carlsbad, CA, USA).
temperature and washed three times with phosphate-buffered saline (PBS) supplemented with protease inhibitors (Roche). Aliquots of the washed beads were run on SDS-PAGE and stained with Coomassie Blue Stain to verify fusion protein integrity.

The preparation of a His-tagged fusion protein expressing amino acids 259-339 of the cytoplasmic domain of CAR has been described previously (35). This fusion protein contains the domains that are common to the two full-length CAR isoforms (m1 and m2 (34)) but lacks the distal most 26 amino acids of the m1 isoform, including the PDZ binding residues (SIV).

GST Pulldown Assay
A 70 – 80% confluent 15 cm^2 or 10 cm^2 plate of U87CAR or 293A cells, respectively was used for affinity binding to GST-CAR. Cell lysates (20mM HEPES, pH 8, 150mM NaCl, 1% Triton X-100, protease tablet cocktail (Roche)) were pre-cleared by incubating with unbound glutathione-Sepharose beads, followed by GST-bound glutathione-Sepharose beads (50µL, 2 hrs each at 4ºC under rotation) to remove any non-specific binding. The pre-cleared lysate was then incubated with 50µL GST-CAR-bound glutathione-Sepharose beads for 2 hrs at 4ºC. The beads were washed three times with 500µL lysis buffer. Interacting proteins were eluted by boiling with 2X Laemmli SDS-PAGE loading buffer containing 5% beta-mercaptoethanol (Bio-Rad, Mississauga, ON, Canada). SDS-PAGE and Western blots were performed as previously described (33). For Coomassie Blue staining, protein polyacrylamide gels were incubated for 1 hr at room temperature with 0.1% Coomassie Brilliant Blue R250 stain (Bio-Rad) in 45% methanol/10% acetic acid and destained overnight in 40% methanol/10% acetic acid.

SDS-PAGE and Western blot
SDS-PAGE and Western blots were performed as previously described (33). For Coomassie Blue staining, protein polyacrylamide gels were incubated for 1 hr at room temperature with 0.1% Coomassie Brilliant Blue R250 stain (Bio-Rad) in 45% methanol/10% acetic acid and destained overnight in 40% methanol/10% acetic acid.

Proteomic Analysis
Following SDS-PAGE, silver staining was performed using a protocol which was compatible with subsequent mass spectrometry. Gels were fixed in 10% acetic acid/50% methanol overnight and washed twice in water for 30 min each. Sensitization was performed for 1 minute with 0.02% thiosulfate followed by two rinses of 20sec with water. Gels were stained in 0.2125% silver nitrate/0.0259% formaldehyde and washed twice for 15sec in water. Development occurred by treatment with 3% potassium / 0.00925% formaldehyde / 0.00125% thiosulfate for 2 min. The reaction was stopped with 3% Tris/ 2% acetic acid for 1min and rinsed twice for 10 min in water. Gels were kept in storage solution of 2% acetic acid and bands were selected for further mass spectroscopy processing. Gel slices were excised and digested with trypsin (6ng/µl) for 5 hrs on a MassPrep robotic workstation (Micromass, Manchester, U.K.). Peptides were extracted in a final volume of 45µl of 0.5% formic acid (v/v) and 9% acetonitrile (v/v). Tryptic peptides were analysed on an LC-QToF (liquid chromatography quadrupole time-of-flight) mass spectrometer (MicroMass). Briefly, the sample was applied to a 10 cm x 75 cm Pico Frit column containing BioBasic C18 packing. Peptides were eluted from the column using a gradient of 10-95% acetonitrile (v/v) containing 0.1% formic acid (v/v) throughout at a flow rate of 200nl/min. Eluted peptides were electrosprayed as they exited the column and doubly or triple charged ions were selected for passage into a collision cell. Fragmentation was induced by collision with argon gas and data collected in 1 second scans for up to 5 seconds. Peaklists of MS/MS data were prepared using Masslynx software (MicroMass) and submitted to Mascot (Matrix Science, Boston, MA, USA) for identification by analysis against the National Center for Biotechnology Information (NCBI) non-redundant database.

Immunoprecipitation Analysis
Cells grown in a 10 cm^2 dish were washed twice in PBS, then scraped into 1ml of immunoprecipitation lysis buffer (IP buffer) [150 mM NaCl, 50 mM TrisHCl, pH 7.4, 1% NP-40, protease inhibitor cocktail (1 tablet, Roche)] and incubated on ice for 30 min. The sample was then centrifuged at 3600 x g_{max} for 15 min at 4ºC. The supernatant was kept and spun again at 17500 x g_{max} in a microfuge at 4ºC for 30 min. The insoluble pellet (which contained the majority of
microtubules) was discarded and the soluble supernatant was then incubated overnight at 4°C with 5 µg of anti-pan β-tubulin mouse monoclonal antibody (Clone Tub 2.1, Sigma (Oakville, ON, Canada) pre-coupled to protein G-Sepharose. Beads were then washed three times with IP buffer and bound protein eluted with 2X SDS-PAGE buffer for Western blot analysis. Blots were incubated with a polyclonal anti-CAR antibody [RP291] which reacts against the cytoplasmic domain of CAR m1 isoform (23) [kind gift of Dr. Kerstin Sollerbrant (Ludwig Institute for Cancer Research, Stockholm Branch, Karolinska Institutet, Stockholm, Sweden)].

**Indirect Immunofluorescence**

Cells were grown in 8-well chamber slides (Nunc, Rochester, NY, USA). Cells were fixed for 15 min with 4% paraformaldehyde in PBS, permeabilized for 5 min with 0.1% Triton X-100 in PBS, and blocked for 30 min with 1% milk/PBS, washing once with PBS between each step. Antibodies RP291 (anti-CAR) and anti-tubulin (Clone Tub 2.1, Sigma) were diluted 1:1000 and 1:2000, respectively, in blocking buffer and incubated overnight at 4°C. Primary antibody was washed off thrice and Alexa 555 goat anti-rabbit and Alexa 488 goat anti-mouse diluted at 1:250 (Invitrogen) were applied for 1 hr at room temperature. After three washes with PBS, the chambers were removed and coverslip applied. Controls consisted of chambers processed similarly but in the absence of primary antibody. Slides were visualized under oil immersion objective (63x) on a Leica DMIRE2 (Richmond Hill, ON, Canada) wide-field fluorescence microscope. The same exposure time was used for all samples. The images were processed using Openlab (Lexington, MA, USA) software. In some experiments, images were collected using a laser scanning confocal microscope (LSM 510, Carl Zeiss MicroImaging Inc.) equipped with argon (488nm) and krypton (568 nm) lasers and a Plan Apochromat 100X/1.4 oil immersion objective lens.

For the nocodazole treatments, cells were plated at a density of 10,000 cells/cm² on coverslips in 24-well tissue culture plates in DMEM supplemented with 10% FBS and antibiotic cocktails for two days. The culture medium was aspirated and replaced with culture medium of the same composition supplemented with 4 µM of nocodazole (Sigma) for 5 minutes at 37°C in 5% CO₂. Cells were then rinsed once with PBS and then with PEM buffer (80mM PIPES, 5mM EGTA, 1mM MgCl₂, pH 6.8) after which cells were incubated with PEM containing 0.5% (w/v) NP-40 and 0.3% glutaraldehyde for 10 min for simultaneous fixation of microtubules and extraction of unpolymerized tubulin heterodimers. After fixation, cells were rinsed with PBS and further permeabilized by incubation with 0.5% Triton X-100 in PBS for 10 min at RT. The cells were washed with PBS and then incubated with 0.1M glycine in PBS for 20 min to quench uncross-linked glutaraldehyde. The cells were then washed once with PBS, blocked in blocking solutions, stained for beta-tubulin and visualized as outlined above. All microphotographs were taken at the same exposure time and magnification.

**Cytotoxicity Assays**

Cells were seeded in 96-well plates at 5000 cells/well. The next day, cells were treated with paclitaxel (Sigma) at 2:3 serial dilutions of 15.8 nM in triplicate. After 72h incubation, cells were fixed and survival calculated with the sulforhodamine B cytotoxicity assay, as previously described (36).

**Tubulin Binding Affinity Measurements**

Purified bovine brain tubulin (Cytoskeleton Inc, Denver, CO, USA) at 10 µM dimer concentration in binding buffer (80 mM PIPES, pH 6.9, 0.5 mM EGTA, 2mM MgCl₂, 1% Triton X-100, 1 mM GTP, 10% glycerol) was placed on ice for 30 min to ensure complete depolymerization. The tubulin sample was then centrifuged for 10 minutes at 10,000 x g to remove insoluble aggregates and then diluted into 250 µl aliquots at different final concentrations from 50 nM to 3.7 µM and added to a fixed amount (10 µl) of GST fusion protein slurry (50%) for 4 hrs at 4°C after which the samples were spun down and washed twice with ice-cold binding buffer. Tubulin bound to the beads was released by SDS-PAGE loading buffer and analyzed by Western blot analysis. The amount of tubulin bound to the GST-fusion protein beads was quantitated against a tubulin standard curve by densitometry using GeneTool software (Syngene, Frederick, MD, USA). A dissociation curve was constructed and the dissociation
constant (Kd) calculated using Prism software (Graph Pad, San Diego, CA, USA).

**Microtubule (MT) Preparation**

Pre-formed MTs used for microtubule binding protein spin down assays, microtubule binding affinity measurements, and electron microscopy were prepared using a microtubule binding protein spin-down assay kit (Cytoskeleton Inc.) immediately before use as per the manufacturer’s instructions. Briefly, 2.5 µl of cushion buffer (80 mM PIPES pH 7, 1mM MgCl₂, 1 mM EGTA, 50% glycerol) was added to 20 µl aliquot of 5mg/ml tubulin protein in general tubulin buffer (GTB) (80 mM PIPES pH 7, 1 mM MgCl₂, 1mM EGTA) and incubated at 37°C. After 20 minutes of incubation, the MTs were diluted with 200 µl of GTB plus 2µl of 2mM paclitaxel for stabilization and the prepared MTs were used at room temperature (RT).

**Microtubule Binding Protein Spin-down Assay**

The ability of fusion protein constructs and test proteins to bind to microtubules was assessed using a microtubule binding protein spin-down assay kit (Cytoskeleton Inc) according to the manufacturer’s instructions. Briefly, 20 µl of taxol-stabilized MT were added to 30 µl purified MAP2, bovine serum albumin (BSA), or purified GST-CAR fusion protein or 6xHis-tagged CAR fusion protein at RT. After 30 min of incubation, the samples were placed on top of 100 µl of taxol-supplemented cushion buffer and centrifuged at 100,000 x g at RT for 40 min in a Beckman Airfuge (Mississauga, ON, Canada). After centrifugation, the supernatants were carefully removed by pipetting off 50 µl from the top. A volume of 12 µl of 5 x SDS-PAGE loading buffer was then added for SDS-PAGE analysis. The cushion buffer was then gently removed from the ultracentrifugation tubes and the microtubule pellets collected by washing the bottom of the tube with 1X SDS gel loading buffer and loaded on gels for SDS-PAGE analysis. For peptide competition assays, an excess of preformed microtubules were preincubated with varying concentrations of peptides for 30 min at RT. The CAR peptide (aa 340-365) was VAAPNLSRMGAVPVMIPAQSKDGIV (MW: 2609; pl: 8.9). The control peptide was CSSRGRNTPGKPMREDTMKLH (MW: 2401; pl: 10). After this preincubation, GST-CAR proteins were added into the mixture and incubated for 30 min as usual before the spindown.

**Microtubule Binding Affinity Measurements**

Purified GST-CAR protein was eluted from glutathione beads using freshly prepared 10 mM reduced glutathione in 50mM TrisHCl buffer (pH 8.0). This was then dialyzed for 24 hours in GTB after which the protein concentration was quantified using the BCA protein assay reagent concentrate as per manufacturer’s instructions (Bio-Rad). The protein sample was diluted into 45 µl aliquots of concentrations ranging from 5.63 µM to 0.115 µM in GTB. A volume of 5 µl of MT prepared as above was then mixed with each of the diluted GST-CAR protein samples and incubated for 1 hr at RT at which time the samples were spun down at 100,000 x g at RT for 40 min on top of a 100 µl taxol-supplemented cushion buffer as in the MT binding protein spin-down assay described above. The MT pellets were then collected and analyzed by Western blot analysis for the presence of GST-CAR protein. The amount of GST-CAR protein that had bound and spun-down with the MTs was quantitated by comparing the intensity of the bands against standard curves using the Syngene computer software. Constant MT quantity in the pellets of all of the samples was also monitored using Western blot analysis. A dissociation curve was constructed and the Kd calculated using Prism software (Graph Pad).

**Electron Microscopy of Microtubules**

For electron microscopic analysis of fusion protein binding to MTs, fusion proteins (GST or GST-CAR) at 5.63 µM were incubated with MTs as in the microtubule binding affinity measurement described above. After incubation, the samples were applied to a carbon-supported copper 300 mesh electron microscope grid (Canemco Inc, Lakefield, QC, Canada), air dried, and counter-stained with lead citrate and uranyl acetate for examination with a JEOL (St-Hubert, QC, Canada) 100 CX transmission electron microscope. The magnification used was 5000X.

**Cell Migration Assays**

Glioma migration was assessed using a Boyden transwell chemotactic assay adapted from those described in (37,38). The transwell chamber consisted of upper chambers with 8µm
polycarbonate filter inserts (Corning, Acton, MA, USA) placed in 24-well tissue culture plates (Nunc). Serum-starved glioma cells (5000 cells), suspended in DMEM containing 1% FBS and 1mg/mL BSA, were seeded into the top compartment of each chamber. In some experiments, U87CAR cells were infected at a multiplicity of 50 with control adenovirus recombinant expressing Blue Fluorescent Protein (AdVBFP) or antisense full-length CAR (AdVAS) (both generated with the AdEasy system [QBiogene, Montreal, QC, Canada]. The bottom well contained U251 glioma conditioned medium to act as directional chemoattractant. In selected experiments, the bottom well was supplemented with 0.5nM paclitaxel (Sigma). After 16 hrs of incubation, cells were fixed with 4% paraformaldehyde in PBS for 30 min at RT, washed twice with PBS, and stained with Hoechst dye (Molecular Probes, Burlington, ON, Canada) diluted 1:10,000 in PBS for 30 min in the dark. Chambers were washed twice with PBS and non-migrated cells were then gently removed from the upper side of the filter insert with a cotton-tipped applicator. The trans-migrated cells remaining on the lower side of the filter insert were then quantified using a Leica wide-field fluorescence microscope with an objective of 40x. For each filter insert, at least 5 random fields were quantified. Each assay was run in triplicate. ANOVA statistical analysis was performed using Prism software (Graph Pad).

RESULTS

Interaction of tubulin with the cytoplasmic domain of CAR. To identify proteins which bind the intracellular domain of CAR [amino acids 259 – 365, m1 isoform (34)], we performed affinity assays using a GST-CAR fusion protein with cell lysates prepared from U87 glioma cells stably expressing the full length CAR [U87CAR (33)]. The lysates were sequentially applied to glutathione-Sepharose and GST-Sepharose to remove non-specific binding, followed by application to GST-CAR-Sepharose. After elution and electrophoretic separation of the GST-CAR binding proteins, proteomic analysis was performed on individual bands that were enriched in the GST-CAR pull-downs as described in the Experimental Procedures. Highly significant hits were seen with multiple peptides of beta and alpha tubulin identified by mass spectrometry as shown in Figure 1.

To validate the results of the proteomic analysis, we verified that the GST-CAR fusion protein could indeed pull down tubulin from U87CAR cell lysates. As shown is Figure 2A, tubulin was detected only in the samples that had been incubated with GST-CAR fusion protein (lane 4) and was absent in samples incubated with either glutathione-Sepharose or GST-Sepharose (lanes 2 and 3 respectively). Similar results were obtained with cell lysates prepared from human embryonic kidney 293A cells which express only endogenous CAR (Figure 2B).

In vivo interaction of CAR with tubulin. To confirm the interaction of CAR with tubulin, we immunoprecipitated tubulin from U87CAR cells with an anti-tubulin monoclonal antibody and analyzed for the presence of CAR by Western blotting (Figure 2C). A portion of the total cellular CAR was present in these immunocomplexes. Moreover, immunoprecipitation of tubulin from human HeLa cells which express endogenous CAR (2) revealed that tubulin and CAR also have the ability to associate in these cells (Figure 2D). Similar co-immunoprecipitation results were obtained in additional cell lines which express endogenous CAR such as the human bladder cancer cell line 253J (31) (data not shown).

CAR is a type I membrane protein. As previously shown, in U87CAR cells, CAR immunoreactivity is detected at the cell surface. When U87CAR cells were immunolabeled with anti-CAR and anti-tubulin antibodies, in all cells examined, CAR immunoreactivity partially overlapped with that of tubulin (Figure 3A). This is consistent with the observation that in tumor cells, the highest tubulin concentration appears in the cell periphery (39). The co-localization was also confirmed by visualization of the immunoreactivity by confocal microscopy (Figure 3B, C). These results are consistent with an interaction of the cytoplasmic domain of CAR with tubulin, either directly or indirectly.
Direct binding of CAR to tubulin heterodimers and microtubules. To test whether CAR can associate directly with tubulin, binding assays were carried out between the GST-CAR fusion protein and purified, depolymerized bovine brain tubulin (> 99% pure) as described in Experimental Procedures. Purified tubulin did not bind GST-Sepharose (data not shown) but it did bind and could be eluted from GST-CAR-Sepharose, indicating that CAR can interact directly with tubulin heterodimers. The binding was saturable (Figure 4). In this assay, the dissociation constant (Kd), as determined by curve fitting with non-linear regression analysis, was ~ 1 µM, a value which is consistent with that obtained for other tubulin binding proteins such as CRMP-2 (0.8 µM) (40) and stathmin (0.5 µM) (41).

We then assessed whether CAR binds microtubules in vitro by performing a microtubule spin-down assay as described in Experimental Procedures. In the presence of pre-formed microtubules, both GST and BSA remained in the supernatant, and did not associate with the microtubules that were found in the pellet after ultracentrifugation (Figure 5A,B). In contrast, GST-CAR fractionated predominantly to the pellet, but only in the presence of microtubules (Figure 5C). The distal portion of the cytoplasmic domain was required for this interaction as a fusion protein lacking the last 26 amino acids of the C-terminus was predominantly found in the supernatant (Figure 5D). As well, a synthetic peptide encoding the distal 26 amino acids competed specifically with GST-CAR binding to microtubules (Figure 5E).

Measurement of the binding affinity of CAR to a constant amount of microtubules was determined using the microtubule spin-down assay shown in Figure 5. CAR bound microtubules more avidly than did the tubulin heterodimer, with a dissociation constant of 0.032 µM (Figure 6A). Furthermore, the interaction of CAR with microtubules resulted in tightly packed arrays as visualized by negative stain electron microscopy (Figure 6B). The higher affinity of CAR for microtubules suggests a possible role for CAR in regulating microtubule dynamics. Incubation of cells with nocodazole, which induces depolymerization of microtubules, results in a time-dependent disruption the microtubule network. Nocodazole treatment, as described in Experimental Procedures, affected CAR expressing cells differentially in that the microtubule network was less susceptible to a 5 min treatment with 4 µM nocodazole in U87CAR cells than in control U87LNCX cells (Figure 6C).

Response of U87CAR cells to paclitaxel. Drugs that interfere with the equilibrium of microtubule assembly are commonly used clinical anti-tumor agents (42). One such drug, the taxane paclitaxel, which shifts the cytoskeleton equilibrium towards assembly of very stable microtubules, has potent anti-glioma activity in vitro (43). To determine if expression of CAR and its interaction with microtubules has an impact on glioma cell response to paclitaxel, we compared cytotoxicity of paclitaxel in U87CAR cells and control vector U87LNCX cells. U87CAR cells had an IC50 of 4.44 +/- 0.28 nM versus an IC50 of 5.65 +/- 0.19 nM for vector control cells (p < 0.0008 by two-tailed t-test; n=3). Taken together with the previous findings on the effect of nocodazole, these results suggest that the association of CAR with microtubules alters microtubule dynamics.

Effect of CAR on glioma cell migration. The cytoskeleton is important for maintaining cell shape and facilitating cell movement (44). A chemotaxis assay in Boyden chambers was used to assess cell motility of U87CAR cells as compared to the vector control U87LNCX cells. Significantly fewer U87CAR cells migrated through the transwell as shown in Figure 7C (LNCX vs CAR). The difference in migration was directly related to the expression of CAR as knockdown of CAR levels to less than 20% by transduction of U87CAR cells with a recombinant adenovirus bearing an antisense construct of full-length CAR (Figure 7A) restored migration to the extent observed in U87LNCX cells (Figure 7C, CAR-AS). Furthermore, the cytoplasmic domain was required for this differential effect as U87 cells expressing on the cell surface a CAR construct that is deleted for the C-terminal domain (33) had similar migration rate as U87LNCX (Figure 7C, CAR 781).
As paclitaxel is known to affect cell migration (45), the Boyden assay was also carried out in the presence of 0.5nM paclitaxel, a concentration which is not cytotoxic to these cells. Although, as expected, paclitaxel inhibited the migration of U87LNCX cells and U87CAR781 cells (data not shown), it had no effect, at this concentration, on the migration of full-length CAR-expressing U87CAR cells (Figure 7D). In the presence of paclitaxel, the difference in the migration rate of U87CAR and U87LNCX cells was abolished, indicating that CAR may affect U87 glioma cell migration through its interaction with microtubules.

DISCUSSION

Compelling evidence is accumulating for CAR being a tumor suppressor (30,32). With increasing malignancy, tumors progressively lose CAR expression, as compared to adjacent normal cells (31,46-49). In tumor cell lines of various origins, CAR overexpression decreases cell proliferation (30,32). Previously, we used retrovirus-mediated gene transfer to express CAR in the U87 glioma cell line at levels equivalent to those found in the developing brain (33). In these cells, CAR expression had a significant impact on growth and invasion in 3-dimensional spheroids, and more importantly, on tumor growth when the cells were implanted into the brain. In both conditions (spheroid and tumor growth), the cytoplasmic domain of CAR was required (33).

In this report we show that one possible mechanism for the observed effects of CAR may be through its interaction with tubulin and microtubules. We have used several different approaches to provide evidence for this binding (pull downs with recombinant protein [Figure 2A,B], co-precipitation from cell lysates that express exogenous as well as endogenous CAR [Figure 2C,D], co-localization in situ [Figure 3]), and have shown that the affinities of the association are comparable to those reported for other tubulin (40,41) or microtubule-binding proteins (e.g. MAP2 (50); myosin-Va (51)) [Figures 4-6].

We also show that the interaction of CAR with tubulin and microtubules is functionally relevant, in terms of drug response to taxanes that interfere with microtubules as well as cell migration. CAR-expressing U87 glioma cells were more sensitive to treatment with paclitaxel, a microtubule stabilizing agent, than control U87LNCX cells. Altered microtubule dynamics can result in microtubule-drug resistance or sensitivity. In particular, decreased microtubule dynamics can manifest itself in increased paclitaxel sensitivity (reviewed in (52)). This suggests that CAR-expression may result in enhanced microtubule stabilization. Although the mechanism for the stabilization is not known, our in vitro observation of increased bundling of microtubules in the presence of GST-CAR suggests a possible way CAR may regulate microtubules. Presumably, the bundling occurs due to the presence of the CAR cytoplasmic domain in the fusion protein. Although GST is known to dimerize and might have contributed to increased crosslinking of the microtubules, control experiments did not reveal any effects of the GST moiety itself (Figure 6B, left panel). However, in this in vitro assay, the dimerization of GST may facilitate the ability of CAR to cross-link microtubules by allowing the CAR domains to dimerize. This may actually mimic the endogenous configuration of CAR since the extracellular domains have been shown to exist as dimers (53) and CAR binds the adenovirus knob as a trimer (54). Similarly, adhesion molecules such as L1 are known to cluster at adhesion sites on the cell surface and to homomultimerize through their ectodomains (55). Therefore, it is conceivable that CAR, as an adhesion molecule, exists as multimers in vivo and is able to cross-link microtubules at adhesion sites. The localized interactions of CAR and microtubules may thus serve to shift the cytoskeletal equilibrium.

To further support this theory, we compared migration of U87LNCX and U87CAR cells in transwell chambers. Microtubules have been shown to control directed cell migration (56). Accordingly, U87CAR cells migrated through a transwell less than control U87LNCX cells or U87CAR781 cells with a deletion of the cytoplasmic domain of CAR (Figure 7C). However, upon microtubule stabilization with a non-cytotoxic dose of paclitaxel (IC50), the migration difference was nullified. It has been
shown that microtubule-binding proteins, such as p27Kip1 (57) and JAM-A (58), can inhibit cellular motility though microtubule stabilization. We propose that CAR may act similarly within a multi-protein complex to reduce microtubule dynamics to minimal basal activity level. Thus when treated with a low dose of microtubule-stabilizing agent, U87CAR cells are not affected, whereas control U87LNCX cells have microtubule dynamics reduced to the same basal level.

Microtubules are known to play important roles in cell migration. For instance, during cell migration, microtubules are targeted to adhesion complexes (59) where they promote cell migration through their contribution to the dissociation of adhesion sites from the substrate. Our data support the theory that CAR may be one such adhesion molecule which can interact with the cell’s cytoskeleton to regulate its function. Through its PDZ-binding domain, it is conceivable that CAR participates in protein complexes that could in turn affect microtubule regulatory molecules.

In conclusion, the demonstration that CAR binds tubulin and microtubules may provide a mechanistic basis for the selective growth advantage imparted by the loss of CAR expression in various tumor types. Additionally, the results potentially implicate this adhesion molecule in protein complexes important for cell proliferation, migration and signal transduction.

References


Footnotes:
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Figure Legends

Figure 1. Identification of tubulin as a CAR-interacting protein by mass spectrometry. The peptides that were isolated after MS/MS analysis are boxed. The Mowse score is also given (significance set at > 32). These hits were considered highly significant because of the Mowse score, the number of peptides matched as well as the sequence coverage obtained for both α- and β-tubulin. In β-tubulin, some peptides occurred twice, with different lengths as indicated [LAVNMVPRLHFFMPGFAPLTSR].

Figure 2. CAR interaction with tubulin. (A,B). Cell lysates (lane 1) from (A) U87CAR and (B) 293A cells were sequentially applied to and eluted from glutathione-Sepharose (lane 2), GST-Sepharose (lane 3) and GST-CAR-Sepharose (lane 4), followed by immunoblotting of the eluted material with an anti-tubulin antibody. Only GST-CAR bound to tubulin. (C,D) Soluble cell lysates (enriched in tubulin heterodimers after pelleting of microtubules) were immunoprecipitated with a monoclonal antibody against tubulin, followed with immunoblotting with a polyclonal antibody against the C-terminus of CAR (RP291). CAR signal was detected only in the anti-tubulin lane and not in the control IgG lane.

Figure 3. Partial co-localization of CAR with microtubules in U87CAR cells. (A) Cells were double stained for β-tubulin and CAR as described in Experimental Procedures. Partial co-localization is seen at the cell membrane in the merged image. (B) Double stained cells were also examined by confocal microscopy. Superimposed, merged pictures are shown in the right panel, with yellow indicating co-localization. A similar extent of co-localization was observed in all U87CAR cells that were visualized (over 50 cells).

Figure 4. Saturation binding analysis of CAR cytoplasmic domain/tubulin heterodimer interactions. Increasing concentrations of purified tubulin (>99% pure) were added to GST-CAR-Sepharose beads as described in Experimental Procedures. The specifically bound tubulin was detected by immunoblotting and quantitated by comparison to a standard curve to derive the saturation binding curve. The dissociation constant was obtained by curve fitting with non-linear regression.

Figure 5. CAR cytoplasmic domain binds assembled microtubules. (A) Characterization of the microtubule spin-down assay. Pre-assembled microtubules stabilized in the presence of taxol (+MT) or buffer lacking microtubules (-MT) were incubated with MAP2, BSA or buffer alone, followed by ultracentrifugation as described in Experimental Procedures. Proteins in the pellet and supernatant were resolved by SDS-PAGE and stained with Coomassie Blue. MAP2 was predominantly found in the pellet in presence of microtubules only (lane 1 as compared to lane 9). BSA was predominantly found in the supernatant (lanes 7 and 10) in the presence (lane 7 as compared to lane 2) or absence of MT (lane 10). Most of the tubulin was found as assembled microtubules in the pellet (lanes 1-3 as compared to lanes 6-8). (B) GST protein was incubated in the presence of pre-assembled microtubules (lanes 1, 3) or buffer lacking microtubules (lanes 2, 4), followed by ultracentrifugation and SDS-PAGE. The majority of the
GST protein was detected in the supernatant fraction (lanes 3,4). (C) GST-CAR fusion protein was processed in the microtubule spin-down assay as described in A, but following SDS-PAGE, the protein was detected by immunoblotting with an anti-CAR antibody. In the presence of pre-assembled microtubules (+MT), GST-CAR was predominantly in the pellet while it remained in the supernatant when ultracentrifuged in the presence of buffer alone (-MT). (D) A His-tagged CAR fusion protein deleted in the last 26 amino acids was processed in the microtubule spin-down assay as described in A. The fusion protein was detected predominantly in the supernatant whether in the presence (+MT) or absence (-MT) of microtubules. P=pellet, S=supernatant. (E) A synthetic peptide containing the last 26 amino acids of CAR or an irrelevant control peptide were preincubated with an excess of microtubules prior to carrying out the spin-down assay as described in Experimental Procedures. There was a dose-dependent decrease in the amount of GST-CAR fusion protein recovered in the pellet fraction only when the preincubation was performed in the presence of the CAR peptide. The experiment was repeated twice.

Figure 6. Analysis of CAR cytoplasmic domain/microtubule interactions. (A) Saturation binding. Increasing concentrations of GST-CAR fusion protein were added to a constant concentration of pre-assembled microtubules as described in Experimental Procedures. The samples were processed in the microtubule spin-down assay as described in Figure 5. The amount of bound CAR was quantitated by immunoblotting with an anti-CAR antibody to generate the saturation binding curve. (B) Electron microscopic analysis of CAR binding to microtubules. Binding of GST protein (left panel) or GST-CAR fusion protein (right panel) was observed by electron microscopy. In the presence of GST, microtubules were seen in single arrays while in the presence of GST-CAR, microtubules appeared more tightly packed. (C) Sensitivity to nocodazole. Cells were treated for 5 min with 0 or 4 µM nocodazole and processed as described in Experimental Procedures. The microtubule network was visualized by immunoreaction with an anti-tubulin antibody and collections of cells were photographed at the same magnification and exposure time. At these experimental conditions, a more extensive microtubule network remained in the U87CAR cells (+noc) than in U87LNCX cells (+noc). The treatment was repeated twice and over 100 cells were visualized each time.

Figure 7. Effect of CAR on glioma cell migration. (A,B) Expression of CAR in various cell lines used in the migration assays. Western blot of cell lysates obtained from (A) U87LNCX, U87CAR, and U87CAR infected with an adenovirus expressing Blue Fluorescent Protein (AdVBFP) or antisense full-length CAR (AdVAS), as well as (B) U87CAR781 cells that express a truncated CAR (amino acids 1 – 260). The blots were immunoreacted with antibody 2240 which recognizes the N-terminal domain of CAR (60). By densitometric analysis, AdVBFP infected U87CAR cells retained 80% of CAR while cells infected with AdVAS had only 20% of initial CAR levels after normalization. (C,D) Chemotaxis in Boyden chambers. Cell migration assays were performed as described in the Experimental Procedures. In (C), U87CAR cells were infected with the recombinant adenoviruses for 48 hrs prior to start of assay. The differences were statistically significant by ANOVA (p < 0.01) and post-hoc Bonferroni analysis on selected comparisons are indicated by *. Infection of U87CAR cells with AdVBFP did not affect migration but decrease of CAR expression through AdVAS infection led to increased migration. (D) U87CAR and control U87LNCX cells were compared in Boyden chambers in the absence or presence of paclitaxel (PTX) as described in the Experimental Procedures. Each experiment was carried out in triplicate and replicated 3 times for the control condition and twice in the presence of PTX. The differences in migration were statistically significant by ANOVA (p <0.004). Post-hoc Bonferroni analysis revealed that under control conditions, migration of U87CAR cells was significantly less than that of the control U87LNCX cells (p <0.01), and that PTX significantly decreased the migration of the control U87LNCX cells (p< 0.001) without affecting the migration of the U87CAR cells (NS; p>0.05).
Beta-tubulin
Total score: 395  Peptides matched: 12
Sequence Coverage: 31%

1 MR(E)IVHIQAG QCGNQIGAKF WEVISDEHGI DPTGTYHGDS DLQLDR(IS)Y
51 YNEATGGK(V)P RAILVDLEP GTMDSVRS(G)P FGQIFRPDNF VFGQSGAGNN
101 WAK( )HYTEGA ELVDSVLDVV REAE5CDCL QGFQLTHSLG GGTGSGMGTL
151 LISKIREEYP DRIIMNTFSSV P3P5KVSDT( )VV EPYNATL5SVH QLVENTDETY
201 CIDNEALYDI CFRTLKLTP TYGDNLNLVS ATMSGVTTCL RFPQNLADLM
251 RKLAVNMVPF PRLHFFMPGF APLTSRGS(Q)Q YRALTVPELT QOVFD( )AKDMMM
301 AACDPRHGRY LTVAAVEFRGR MSMKEVDEQM LNVQNK( )SSY FVEWIPNNVK
351 TAVCDIPPRG LKMAVTIGN STAIQELFKR ISEQFTAMFR RKAFLKVYGT
401 EGMDEMEFTE AESNMNDLVS EYQQYQDATA EEEEDFGEAA EEEA

Alpha-tubulin
Total score: 208  Peptides matched: 7
Sequence Coverage: 22%

1 MRECISIHVG QAGVQIGNAC WELYCLEHGI QPDGQMPSDK TGGGDDSFN
51 TFFSETGAKH HPRAVFVFDL EPTVIDE(V)RT GTYRQOLFHPE QLITGKE( )AA
101 NN( )YARNHYTI GE( )IIDLVLD RIRKLADQCT RLQGFVLFRH FGGTGSFGFT
151 SLLMERLSVD YGKKSKLEFS T3YPAPQVSTA VVEPYNSILT THHTLEHSDC
201 AFMVDNEAIY DICRRNLDIE RPTYTNLNRL IGQIVSSITA SRLFDGALNV
251 DLTFEQTNLV PYPR( )IHFPLA TYAPVISA( )EK AYHEQL( )SVAE ITNACFEPAN
301 QMVKCDPGGH KYMACCLLYR GDVVPKDVNA AIATIKTK( )K IQFVDWHCP( )T
351 FKVGINSYQP TVVPGGDLAK VQR( )AVCMLSN TTAIAEAWAR LDHKFDLM( )YA
401 KRAFVHWYVG EGMEEG(E)FSE AR( )DMAALEK DYEEVGHSV EGEGBEEE( )E
451 Y
The Coxsackie and adenovirus receptor (CAR) binds microtubules and plays a role in cell migration

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