Coxsackievirus and Adenovirus Receptor (CAR) Is Modified and Shed in Membrane Vesicles†

Steven D. Carson*

Department of Pathology and Microbiology, University of Nebraska Medical Center, 986495 Nebraska Medical Center, Omaha, Nebraska 68198-6495

Received January 30, 2004; Revised Manuscript Received April 13, 2004

ABSTRACT: Vesicles shed by U87-MG cells contain coxsackievirus and adenovirus receptor (CAR) protein that has been posttranslationally modified. Relative to full-length CAR, migration of the vesicle-associated soluble CAR antigen (CARd6) on SDS−polyacrylamide gels indicated a loss of approximately 6 kDa. HeLa and END-HHV6 cells also shed a similar vesicle-associated CAR protein. Vesicles shed by U87-MG cells following stimulation with calcium and A23187 contained CARd6 similar to that present in vesicles shed constitutively. RD cells transfected to express full-length CAR produced CARd6, but cells that expressed CAR with a truncated cytoplasmic domain produced no equivalent to CARd6. In U87-MG cells, calpain activity was required for release of CARd6 with shed vesicles, and accumulation of CARd6 in cells that rounded up and released from the plastic substrate in response to A23187 treatment was blocked by *N*-ethylmaleimide. These experiments show that CAR, posttranslationally modified in the cytoplasmic domain, can be released with vesicles shed by cells. Posttranslational modification of the CAR cytoplasmic domain occurs during cell rounding and release from the culture substrate. This modified, vesicle-associated CAR was the principal form of soluble CAR released by the cells.

Membrane vesicles shed from cells are of particular interest as markers of cell activation, as well as for their interactions with cells dissimilar to those from which they originate. Circulating vesicles derived from activated platelets and endothelial cells have been used as markers of inflammation, active coagulation, and endothelial cell damage. They have been studied with particular regard to prothrombotic pathology in cancer and tumor evasion of the immune response and appear to be vehicles by which membrane components are exchanged among cells (see ref *1* for a recent review).

Ultrastructural investigations suggested that tight junction elements of cultured cells can be sequestered into membrane blebs and subsequently shed into the culture medium (*2*). The coxsackievirus and adenovirus receptor (CAR),[1] an immunoglobulin superfamily protein that normally functions as a cell adhesion molecule (*3*−*5*), has been localized to cell junctions (*6*) and shares structural properties with junctional adhesion molecules (JAMs) (*7*, *8*). CAR might therefore be released with vesicles that include tight junction elements, thereby producing a "soluble" pool of CAR that remains membrane-associated.

Experiments reported here show that U87-MG cells, which constitutively shed vesicles in culture (*9*), release "soluble" CAR associated with the shed vesicles. The results further show that the vesicle-associated CAR is a posttranslationally modified form of full-length CAR.

EXPERIMENTAL PROCEDURES

U87-MG cells, a glioblastoma cell line, RD cells, a rhabdomyosarcoma cell line, and HeLa cells were acquired from ATCC. END-HHV6 is a cell line derived after human herpes virus-6 infection of human umbilical vein endothelial cells (*10*). The RD-CAR cell line, generated by transfection of RD cells with CAR cDNA in pcDNA3.1, expresses full-length CAR (pcDNA3.1-CAR kindly provided by Dr. Joann Douglas and Dr. Victor Krasnyck, University of Alabama, Birmingham), as previously described (*11*). RDt3 cells express wild-type extracellular and transmembrane domains of CAR with a modified cytoplasmic domain (includes the first four native amino acids followed by 39 residues encoded by the pcDNA3.1 polylinker)(*12*). Cells were grown in DMEM (high glucose) with 10% fetal calf serum and supplemented with glutamine, penicillin, streptomycin, and gentamicin (Invitrogen/GIBCO-BRL). Calpeptin was from CalBiochem. Other reagents were from Sigma Chemical Co. or Fisher Life Sciences, unless otherwise noted.

Vesicles shed into the culture media were isolated much as previously described (*13*, *14*). Conditioned medium was collected, centrifuged (15 min, 834 × *g*) to remove cells and heavy particles, and concentrated on an Amicon XM300 ultrafiltration membrane. When used, phenylmethylsulfonyl fluoride (PMSF) and *N*-ethylmaleimide (NEM) were added to 2 and 5 mM, respectively, from a 40× stock in 100% ethanol. The concentrate was applied to a column (1 cm × 17 cm) of Sephacryl S1000 and eluted with TBS (0.05 M Tris, 0.1 M NaCl, pH 7.6, with 0.02% sodium azide).

† This work was partially supported by grants from the American Heart Association (Grant 0350705Z) and the NIH (Grant AI054551).

* Phone: 402-559-4710. Fax: 402-559-4077. E-mail: scarson@unmc.edu.

[1] Abbreviations: CAR, coxsackievirus and adenovirus receptor; DMEM, Dulbecco's minimal essential medium; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; FCS, fetal calf serum; HRP, horseradish peroxidase; JAM, junctional adhesion molecule; NEM, *N*-ethyl maleimide; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline; TF, tissue factor.

10.1021/bi049778d CCC: $27.50
Published on Web 06/03/2004

Fractions were assayed for protein (BCA assay, Pierce Chemical Co.) and for tissue factor activity (*15*), a marker associated with membrane vesicles shed by U87-MG cells (*9*). Fractions with peak tissue factor activity were pooled, and proteins were precipitated with 5 volumes of cold acetone (−20 °C overnight, followed by centrifugation at 1876 × *g* for 30 min). Precipitates were dissolved in 1× SDS sample solvent with 20 mM dithiothreitol (DTT), sonicated (Branson sonicator bath) to disperse the precipitates, and heated in boiling water in preparation for analysis by SDS−polyacrylamide gel electrophoresis (10% or 10−15% polyacrylamide) and Western blotting(*16*, *17*). Apparent molecular weights were calculated relative to prestained marker proteins (Amersham or BioRad). CAR was detected on the blots using MoAb.E1 (*18*) and rabbit anti-mouse IgG-HRP (DAKO, absorbed to reduce cross-reactivity). Blots were developed with ECL+Plus (Amersham) and visualized on RX-G film (www.clinicalfilm.com). Images of gels and blots were acquired with either a flatbed scanner (HP Scanjet 5370C) or a Canon 10D camera. Images were adjusted for white balance and levels and prepared for publication in Adobe PhotoShop.

For A23187 induction of vesicle shedding, conditioned medium was replaced with fresh DMEM (with or without FCS, as indicated) to which Br-A23187 in DMSO (final concentration 20 μM) or DMSO alone was added. Cells were incubated (37 °C) for 15 min or until cells began to round up and release from the plastic (for U87-MG, 10−12 min in the presence of FCS and 4−7 min when FCS was absent). Vesicles were isolated from the culture media as described above. When sloughed cells were studied, the cell pellet obtained from the first centrifugation of the vesicle isolation procedure was lysed in TBS with 2% octyl glucoside and cleared by centrifugation (15 min, 1876 × *g*), and soluble proteins were precipitated with acetone prior to dissolution in SDS sample solvent. Adherent cells were scraped into TBS, pelleted in the centrifuge (15 min, 834 × *g*) and lysed with TBS with 2% octyl glucoside. Lysates were collected after centrifugation (15 min, 1876 × *g*), and proteins were precipitated with acetone prior to dissolution in SDS sample solvent. When used, calpeptin (final 570 nM) was added to the medium on the cells. After 15 min at 37 °C, the medium was replaced and additional test reagents (e.g., A23187) were added. Pretreatments with NEM (5 mM) or PMSF (2 mM) were limited to 5 min.

RESULTS

U87-MG cells are readily infected by group B coxsackieviruses, and CAR expression has been confirmed by Western blotting (S. Tracy, N. Chapman, and S. Carson, unpublished) (*19*). U87-MG cells also express tissue factor (TF), which is shed into the culture medium with membrane vesicles (*9*). These vesicles are readily isolated from conditioned culture medium by chromatography on Sephacryl S1000 using TF activity as a marker for the vesicles (Figure 1). The fractions containing the peak TF activity (fractions 6−8 in Figure 1) were pooled, acetone-precipitated, and analyzed for CAR by Western blotting. The blots (Figure 1) showed that CAR was present in the vesicles but migrated with less apparent mass than CAR from the adherent U87-MG cells. CAR was not detected in fractions lacking TF activity. This experiment thus identified a "soluble" (i.e., shed into the medium) form of CAR that cochromatographed with membrane vesicles. The blots shown in Figure 1 are particularly striking in that the parental CAR (lower mobility) was the predominant antigen detected in the adherent cells, whereas the higher mobility CAR predominated in the vesicles. The apparent molecular masses of the two distinct CAR antigens (estimated from 15 Western blots) were found to be 43.6 ± 4.6 kDa and 37.5 ± 3.4 kDa. The mass difference determined from the individual blots was 6.1 ± 1.9 kDa, and the vesicle-associated, smaller form of CAR is referred to as CARd6 to reflect the apparent loss of about 6 kDa.

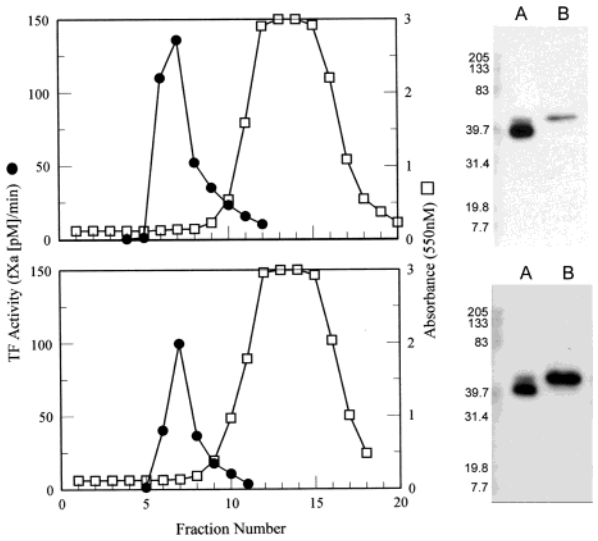

FIGURE 1: Modified CAR is present in vesicles isolated from U87-MG-conditioned medium. Sephacryl S1000 elution profiles of vesicles shed into the medium by U87-MG cells are shown in the left panels. In the two experiments shown, the protein content of the vesicle pools (fractions 6−8) was <1 μg. Western blots (right panels) show that CAR is present in both the vesicles (lanes A; $^1/_3$ of sample loaded, top; $^1/_{10}$ of sample loaded, bottom) and adherent cells (lanes B; 7 μg loaded, top; 9 μg loaded, bottom). The increased electrophoretic mobility of the vesicle-associated CAR corresponds to a decrease of about 6000 M_r relative to the cell-associated CAR. The blots were developed to achieve similar band intensities in the A lanes. The difference in B lane loading relative to the A lane loading differed about 6-fold between the two experiments.

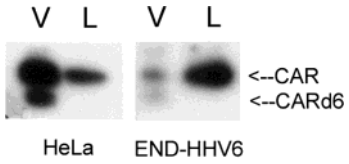

FIGURE 2: HeLa and END-HHV6 cells also shed vesicle-associated CARd6 into the medium. Vesicles were isolated from media conditioned by confluent cultures of HeLa and END-HHV6 cells grown in T75 flasks, and the adherent cells were harvested and lysed. Western blot analysis shows that the isolated vesicles (lanes V) contained CAR and CARd6, whereas only CAR was detected in the adherent cell lysates (lanes L).

To determine whether shedding of vesicle-associated CARd6 was unique to U87-MG cells, vesicles were isolated from media collected from cultured HeLa cells and END-HHV6 cells. Western blot analyses of the isolated vesicles and lysates of adherent cells (Figure 2) showed that the vesicles shed by these cell lines also contained CARd6, as well as the full-length CAR protein and that only full-length CAR was detected in lysates of the adherent cells.

Calcium and ionophore A23187 stimulate vesicle shedding from a variety of cell types, including U87-MG cells, and this stimulation provides a mechanism to generate useful amounts of vesicles over a short time period (*13*). To compare CAR in spontaneously shed vesicles to CAR in vesicles induced by calcium and ionophore, U87-MG cells were grown to high density over several days, and the medium was collected for isolation of spontaneously shed vesicles. Shortly after adding fresh medium (with 10% FCS), separate cultures were untreated, treated with DMSO, or treated with A23187. Cells began to round up and release from the plastic within 15 min of treatment, and medium was gently collected and processed for isolation of vesicles. Adherent cells were also harvested for analysis of cell-associated CAR. As shown in Figure 3, the A23187-treated cells shed TF-rich vesicles into the medium, while vesicular TF activity was nearly undetectable in the untreated and DMSO-treated cultures. Western blot analysis showed that CARd6 was the predominant form of CAR present in vesicles isolated from the pretreatment conditioned medium as well as in the vesicles isolated from the A23187-treated culture. No CAR or CARd6 antigen was detected in the corresponding fractions from untreated and DMSO-treated cultures. The combined low TF activity and lack of CAR antigen in these control samples indicate that few vesicles were present (TF activity can vary among vesicle preparations with influences including cell density and time after treatment with A23187 (*13*, *14*)). While small amounts of CARd6 antigen were detected in the adherent cells, the 43.6 kDa CAR antigen was predominant. These results show that CARd6 is the predominant CAR antigen associated with shed vesicles, irrespective of whether the vesicles were shed constitutively over several days or rapidly in response to calcium and ionophore stimulation, and is generated relatively quickly (<15 min) in cells treated with A23187.

As noted above, U87-MG cells round up and release from the plastic following treatment with A23187. Examples of the morphological response are shown in Figure 4. With respect to gross morphology and shedding of vesicles, the U87-MG cell responses to A23187 are similar to those reported to occur in similarly treated fibroblasts (*13*).

Cells expressing recombinant CAR were used to determine whether the modification that converts CAR to CARd6 involves the cytoplasmic domain. U87-MG cells, RD-CAR cells, and RDt3 cells were tested for CAR modification and shedding following treatment with A23187. In these experiments, serum was omitted during the A23187 treatment so that vesicle-associated proteins could be rapidly isolated by acetone precipitation after removal of cells by centrifugation. A23187 caused a rapid cellular response in the absence of FCS, and more cells rounded up and released from the plastic than in experiments with serum present (see Figure 4). Therefore, sloughed cells were also analyzed for CAR and CARd6. The adherent cells after treatment with DMSO contained the parental CAR antigen (43.6 kDa in U87-MG and RD-CAR; 36.5 kDa in RDt3) (Figure 5A). The adherent RD-CAR cells also contained a significant amount of CARd6, possibly due to the engineered overexpression of CAR in these cells. After A23187 treatment, CARd6 was increased relative to CAR in the adherent U87-MG cells and RD-CAR cells, possibly due to many cells that rounded up but remained adherent. RDt3 cells showed only the CARt3

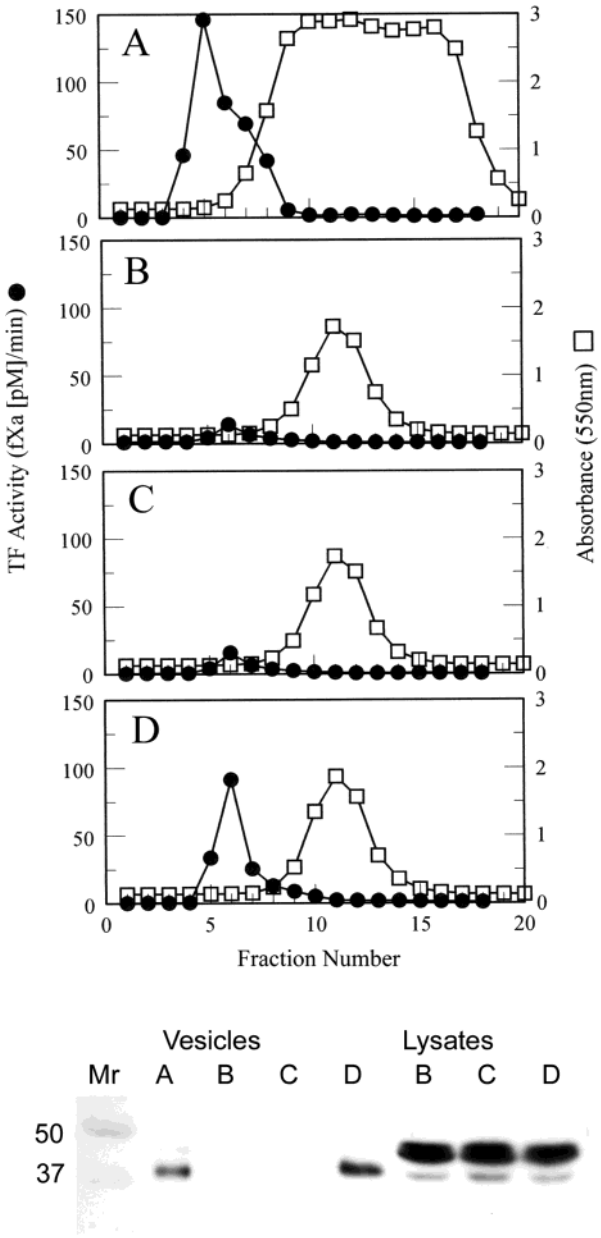

FIGURE 3: Vesicles shed in response to A23187 contain CARd6 similar to that in spontaneously shed vesicles. Sephacryl S1000 elution profiles of vesicles from (A) medium accumulated during culture and from cells 12 min after treatment with (B) fresh medium, (C) fresh medium and DMSO, and (D) fresh medium and Br-A23187 are presented. The Western blot shows that CAR associated with vesicles produced following Br-A23187 treatment (vesicles, lane D) is indistinguishable from that associated with vesicles shed spontaneously (vesicles, lane A). The lysates of adherent cells contain similar CAR, and some CARd6, irrespective of treatment (lysates, lanes B, C, and D).

antigen, with no evidence of antigen with greater mobility (i.e., no CARt3d6). Few cells sloughed off of the plastic in the control samples, and no CAR antigens were detected in these samples (Figure 5B, no A23187). U87-MG and RD-CAR cells sloughed into the medium during the A23187 treatment contained notably more CARd6 than CAR, but only the parental CARt3 antigen was present in the sloughed RDt3 cells. The media samples, which contained the shed vesicles, as well as any cell debris that failed to pellet during centrifugation, contained no demonstrable CAR in the untreated cultures, consistent with the paucity of loose cells

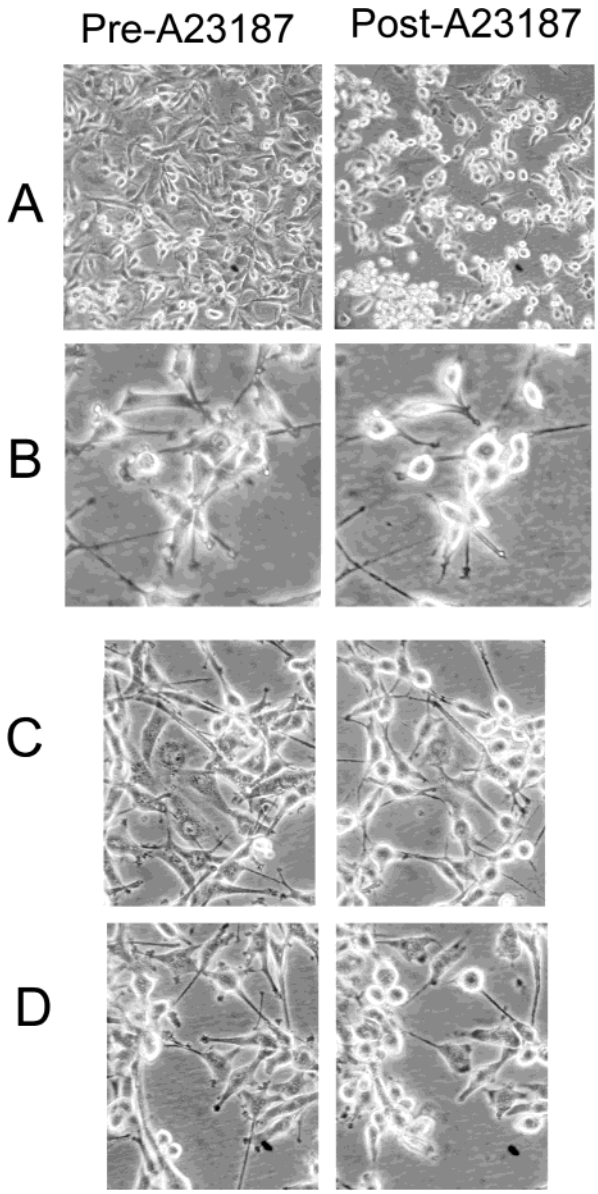

FIGURE 4: U87-MG cells round up following treatment with Br-A23187. Panel A shows that confluent U87-MG cells (left) round up and release from the plastic at 22 min after treatment with A23187 (right). Panel B presents an enlarged view of U87-MG cells in a single photographic field before (left) and 15 min after (right) treatment with A23187. Panels C and D show single photographic fields of U87-MG before and 15 min after treatment with A23187 in the presence (C) and absence (D) of FCS.

and vesicles in these samples (Figure 5C, no A23187). The media from A23187-treated U87-MG and RD-CAR cells contained predominantly CARd6, whereas only the parental CARt3 was detected in medium from the A23187-treated RDt3 cells. This result shows that the modification of CAR that produces CARd6 must occur in the cytoplasmic domain, since CAR and CARt3 differ only in their cytoplasmic domains. Moreover, assuming that translation does not stop prematurely, the CARd6 must be a posttranslational modification since the parental CAR is expressed from cDNA with no introns.

To determine whether calpain might mediate CARd6 production and shedding with vesicles, the effect of calpeptin was examined. Irrespective of whether they were treated with calpeptin, A23187-treated U87-MG cells shed vesicles with

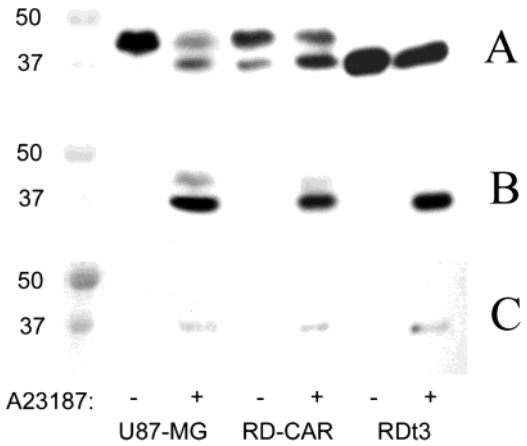

FIGURE 5: Comparison of CAR to a variant with truncated cytoplasmic domain shows that the CARd6 modification requires the cytoplasmic domain. Western blot analysis of CAR in lysates of adherent U87-MG, RD-CAR, and RDt3 cells (A), sloughed cells (B), and CAR remaining in the medium after centrifugation (C), following treatment with or without Br-A23197 (in the absence of FCS). Only cells expressing CAR with native full-length cytoplasmic domain (U87-MG and RD-CAR) had modified CAR (CARd6) associated with cells (adherent or sloughed) or shed into the media.

comparable tissue factor activities (Figure 6), but CARd6 was detected only in the vesicles from cells stimulated with A23187 (in medium with serum) in the absence of calpeptin. No CAR or CARd6 antigen was detected in the S1000 fractions from the DMSO or calpeptin alone control samples or in the vesicles from the sample treated with calpeptin prior to A23187. This result showed that, although calpeptin failed to block shedding of vesicles containing tissue factor, it did block incorporation of CARd6 into those vesicles. CAR was the predominant antigen in the adherent cells, though CARd6 was detected in all of these samples. The absence of CARd6 in the vesicles induced by A23187 in the presence of calpeptin, despite the presence of the small amount of CARd6 in the adherent cells, shows that calpeptin blocked incorporation of CARd6 into the vesicles.

The U87-MG cells that release from the plastic during treatment with A23187 represent a significant proportion of the total cells, especially in the absence of serum, and therefore contain a pool of CAR, or CARd6, not yet examined with regard to calpeptin effects. Experiments were therefore repeated to include analysis of all cells, as well as vesicles, following treatment with calpeptin (added to medium with serum) and A23187 (in the absence of serum). Western blots showed that adherent DMSO-treated cells contained predominantly CAR (Figure 7). DMSO caused few cells to slough from the plastic and no measurable vesicle shedding, and no CAR was detected in lanes loaded with those samples. The adherent cells treated with A23187 contained predominantly CARd6 and diminished amounts of total CAR antigen relative to levels expected from preceding experiments (A23187 treatment in the presence of serum), irrespective of calpeptin. Much of the CAR antigen, as CARd6, from the A23187-treated samples was present in the sloughed cells with no apparent differences attributable to calpeptin. On the other hand, vesicle-associated CARd6 was diminished in the cells treated with calpeptin and A23187 relative to that detected in cells treated only with A23187. These results show that 570 nM calpeptin

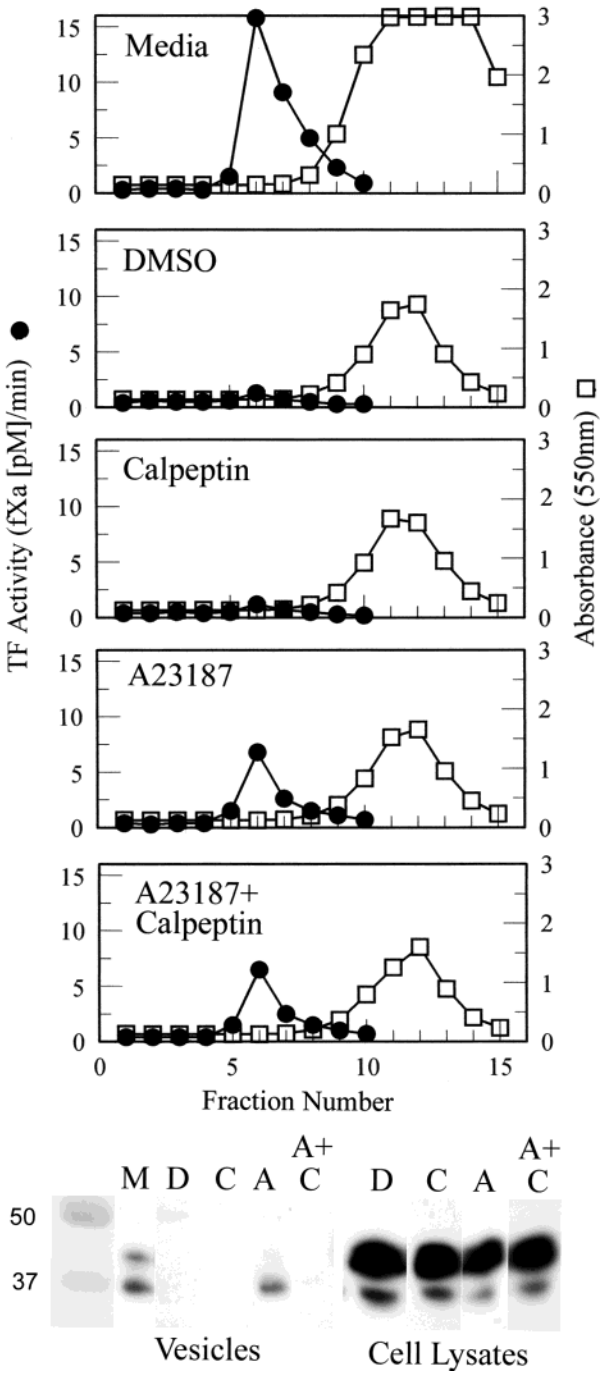

FIGURE 6: Effect of calpeptin on vesicle production and CAR modification induced by A23187 treament of U87-MG cells. The Sephacryl S1000 elution profiles show that calpeptin alone (570 nM) did not stimulate production of vesicles with TF activity and A23187-induced vesicles contain equivalent levels of tissue factor activity irrespective of the presence of calpeptin. Western blots showed that neither DMSO alone (lane D) nor calpeptin alone (lane C) resulted in U87-MG shedding of CAR in the vesicle fractions (consistent with lack of TF activity shown on the elution profiles). CARd6 was present in the vesicles induced by A23187 (lane A), but absent from vesicles induced by A23187 in the presence of calpeptin (lane A+C). CAR and CARd6 detected in the adherent cells were similar irrespective of treatment.

inhibited inclusion of CARd6 into A23187-induced shed vesicles, but with A23187 added in the absence of serum, calpeptin did not block conversion of CAR to CARd6, most of which was present in the cells that rounded up and released from the culture surface.

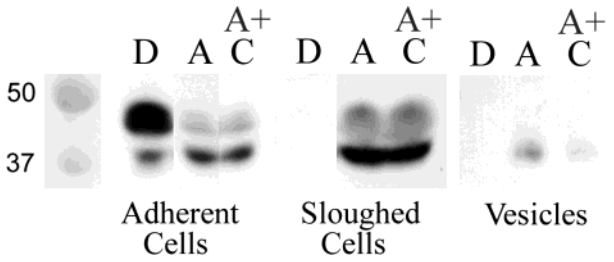

FIGURE 7: Calpeptin inhibited the shedding of CARd6 in vesicles induced with A23187 in the absence of fetal calf serum but did not block the conversion of CAR to CARd6, most of which appears in the cells that rounded up and sloughed from the culture surface. The blots show that CAR is present in the lysates of DMSO-treated cells (lane D). Cells did not slough off of the culture surface and did not shed vesicles when treated with DMSO, and no CAR was detected in those samples. In the adherent cells after treatment with A23187 (lane A) or calpeptin and A23187 (Lane A+C), CAR was diminished relative to the DMSO-treated controls, and CARd6 appeared to be increased. The blot of vesicle fractions showed that CARd6 was present in the A23187-induced vesicles but detected at only low level in the vesicles induced by A23187 in the presence of calpeptin. Most of the CAR antigen in the sloughed cells, which were present in significant amounts after A23187 treatment, was CARd6.

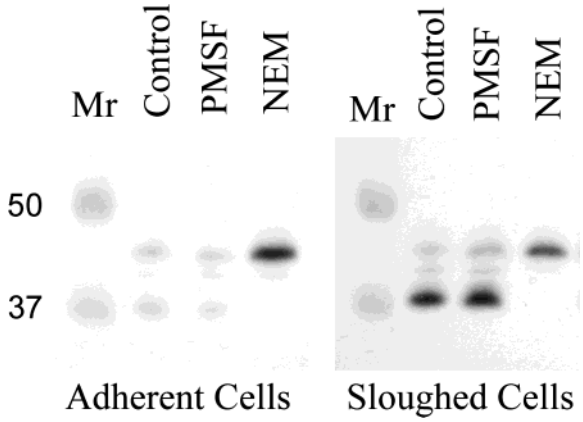

FIGURE 8: NEM inhibited the conversion of CAR to CARd6. Most U87-MG cells treated with A23187 (control lane) sloughed off of the culture surface, and most of the CAR antigen (CARd6) was present in that sample. When treated with A23187 in the presence of PMSF, results were no different than control. When treated with A23187 in the presence of NEM, more cells remained adherent and CARd6 was not detected in either the adherent or sloughed cell populations.

Additional experiments tested whether phenylmethylsulfonyl fluoride (PMSF), an inhibitor of serine proteases, or *N*-ethylmaleimide (NEM), an inhibitor of cysteine proteases due to its reactivity with free sulfhydryls, would inhibit the conversion of CAR to CARd6 in the cells that round up and slough into the medium when treated with A23187. U87-MG cells were treated with A23187 alone, with A23187 after PMSF, or with A23187 after NEM. Except in the sample treated with NEM, most of the cells sloughed from the plastic, leaving few adherent cells for analysis. The few adherent cells remaining after treatment with A23187 or PMSF and A23187 contained low but detectable amounts of both CAR and CARd6 (Figure 8). The NEM and A23187-treated adherent cells contained readily detectable CAR with no CARd6 detected. Sloughed cells from the control (A23187 alone) and PMSF plus A23187-treated cultures contained predominantly CARd6. Sloughed cells from the NEM-treated culture contained CAR, but no detectable CARd6. This result

shows that the conversion of CAR to CARd6 was not affected by PMSF but was inhibited by NEM. It also establishes that cells can slough from the plastic in the absence of CAR conversion to CARd6.

DISCUSSION

In addition to its normal function in cell−cell junctions (*6*), CAR is a principal determinant of coxsackievirus and adenovirus tropism and therefore the primary target for these pathogenic viruses as well as for adenoviruses adapted for use in gene therapy (*20*, *21*). Soluble CAR produced through alternative mRNA processing may interfere with infection by adenoviruses, including adenovirus-based vectors (*22*). More recent work has also shown that cells engineered to express alternatively spliced forms of CAR, identified by RT-PCR, can secrete CAR isoforms that inhibit infection by coxsackievirus (*23*). The results reported here show that vesicle-associated (soluble) CAR can be readily detected in media used to culture U87-MG, HeLa, and END-HHV6 cells that have not been genetically engineered for expression, as well as in RD cells engineered to express full-length CAR or CARt3. This vesicle-associated CAR may also be able to interfere with infection by coxsackieviruses or adenoviruses. Indeed, the soluble CAR detected in malignant pleural effusions, shown to inhibit adenovirus infection (*24*), may have been vesicle-associated. Although not yet examined, the presence of CAR may also impart specific adhesive properties to the vesicles, thus influencing their interactions with cells as well as viruses.

The results reported above show that CAR can be posttranslationally modified and shed from cells as a vesicle-associated "soluble" form of CAR. The vesicle-associated CAR exists predominantly as a form that migrates with greater mobility on SDS−PAGE, corresponding to a loss of about 6 kDa. The CARd6 results from posttranslational modifications to the full-length protein, rather than from translation of alternative transcripts, and is produced by cells that express full-length CAR. CAR lacking the wild-type cytoplasmic domain is not processed to an equivalent lower molecular weight form when expressed in RD cells, showing that the posttranslational modification is dependent on the CAR cytoplasmic domain.

The relative amounts of CAR and CARd6 differ among adherent cells, rounding or sloughed cells, and vesicles. Initial experiments with U87-MG cells (e.g., Figure 1) suggested a nearly exclusive partitioning of CARd6 into vesicles with only CAR remaining in the adherent cells. Subsequent results showed that, while full-length CAR is generally the principal form present in adherent cells, some CARd6 can also be present. And, although CARd6 is the principal form found in vesicles, full-length CAR can also be present in the vesicles. The partitioning of CAR and CARd6 is less distinct for the RD-CAR, HeLa, and END-HHV6 cell lines, though CAR remains the most abundant form detected in adherent cells not treated with A23187.

After ionophore treatment, CARd6 is the form most prevalent in adherent RD-CAR cells, is elevated in U87-MG cells, and is the principal form found in sloughed cells and vesicles. These relationships suggest that CAR is modified to CARd6 as contacts among cells and between cells and the culture substrate are dismantled in the processes required to release vesicles into the medium or for cells to round up and detach. Since the apparent 6 kDa difference between CAR and CARd6 is localized to the cytoplasmic domain, the carboxyl-terminal PDZ domain recognition motif is probably absent in CARd6. Without this motif, the capacity for CARd6 to associate with PDZ proteins (e.g., ZO-1, (*6*)) would be lost, thus freeing CAR and other junctional proteins from this potential constraint to redistribution.

Calpeptin did not prevent shedding of vesicle-associated tissue factor from cells treated with A23187, but shedding of vesicle-associated CARd6 was inhibited by 570 nM calpeptin. Calpeptin did not prevent conversion of CAR to CARd6, which accumulated in cells that rounded and sloughed into the medium. Production of CARd6 was, however, blocked by NEM, which is widely used to inhibit cysteine proteases due to its reactivity with cysteine (*25*). PMSF, a broad inhibitor of serine proteases (*26*) did not inhibit CARd6 production, but L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK) protected about 50% of the CAR from conversion (one experiment, not shown). While generally used as an inhibitor of chymotrypsin-like serine proteases, TPCK can also inhibit cysteine proteases, including calpain (*27*). These results show that shedding of CARd6 in vesicles is sensitive to a specific calpain inhibitor, while conversion of CAR to CARd6 is sensitive to a nonspecific but highly effective inhibitor of cysteine proteases.

μ-Calpain activation occurs in consecutive steps, and the intermediate and mature forms have different sensitivities to calpeptin as well as different substrate specificities. Low concentrations of calpeptin inhibit generation of the mature form of activated μ-calpain but not generation of the activated intermediate (*28*). Consistent with the report by Schoenwaelder et al. (*28*), Basse et al. had previously shown that vesicle formation and proteolysis of cytoskeletal proteins in A23187-treated platelets displayed differential sensitivities to calpeptin concentration (*29*). NEM is expected to broadly inhibit cysteine protease activity, including that of the intermediate calpain activation product. Accordingly, the ability of calpeptin to block CARd6 inclusion into vesicles implicates calpain in this process. Failure of calpeptin to inhibit CARd6 accumulation does not eliminate calpain as a mediator of this step, especially since it is sensitive to other inhibitors of cysteine protease activity.

The results identify distinct events associated with CAR shedding in vesicles. The CAR cytoplasmic domain is modified, probably by a cysteine protease that is sensitive to NEM but not to 570 nM calpeptin. The resulting CARd6 may accumulate in rounding and sloughed cells, or in a calpeptin-sensitive process, CARd6 incorporates into membrane domains that give rise to shed vesicles. Conversion of CAR to CARd6 precedes the calpeptin-sensitive step, so the calpain substrate, possibly proteins of the cytoskeleton (*29*), that controls whether CARd6 will be retained in rounded cells or incorporated into vesicles remains to be identified.

While many details of the molecular mechanisms of CAR conversion to CARd6, as well as mechanisms involved in formation and shedding of vesicles, remain to be elucidated, results presented here clearly demonstrate that "soluble" CAR can be shed by cells, and most of the shed CAR is modified to CARd6 through posttranslational mechanisms. The vesicle-

associated CAR is readily detectable in media from cultured cells that have not been modified to express putative products of alternative transcripts.

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BI049778D