Limited proteolysis of the coxsackievirus and adenovirus receptor (CAR) on HeLa cells exposed to trypsin

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Received 6 September 2000; accepted 10 October 2000

First published online 19 October 2000

Edited by Hans-Dieter Klenk

Abstract Trypsin treatment of HeLa cells results in a limited proteolysis of the coxsackievirus and adenovirus receptor (CAR) after which the cleaved CAR remains cell-associated and tryptic peptides remain associated through disulfide bonds. Trypsin-treated HeLa cells remain susceptible to infection with coxsackievirus B and produce progeny virus at 8 h post-infection in amounts comparable to cells with intact CAR. HeLa cells remove the proteolysed CAR within 15 h and require over 24 h to restore intact CAR to control levels. As turnover is relatively slow, physiological functions that require intact CAR protein may be compromised for more than 24 h following trypsin treatment. Moreover, since removal of proteolysed CAR proceeds at more than twice the replacement rate, trypsin treatment disrupts the receptor-per-cell steady state for at least 24 h.

Key words: Coxsackievirus; Adenovirus; Receptor; CAR

1. Introduction

B group coxsackieviruses (CVB) and adenoviruses share a single protein as the cell surface viral receptor (coxsackievirus and adenovirus receptor; CAR) [1–3]. The CAR protein has been well characterized at the molecular level, including the structure of the Ad-binding domain associated with the Ad fiber knob [4]. There is scant information regarding CAR cell biology, however, and the physiological function of CAR is unknown. Since CAR is a principal factor involved in gene therapy efforts using Ad2- and Ad5-based vectors, as well as efforts to develop CVB-based vaccines, knowledge of CAR cell biology and function is fundamentally important to future applications of these viruses.

Experiments conducted before isolation of CAR found that the binding of CVB to HeLa cells is not altered by trypsin treatment [5–7], and in vitro experiments often use cultured cells exposed to virus only hours after subculture with trypsin. The experiments reported here reveal that CAR is not resistant to trypsin, and treatment of HeLa cells with trypsin alters the expression of this viral receptor.

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Abbreviations: CAR, coxsackievirus and adenovirus receptor; CVB, coxsackievirus B; Ad, adenovirus

2. Materials and methods

HeLa cells were grown in DMEM with 10% fetal calf serum, glutamine, penicillin, streptomycin, and gentamicin. To determine the effects of trypsin on CAR, confluent cell monolayers in T25 flasks were rinsed (×3) and covered with 2 ml DMEM. Trypsin (TPCK trypsin, 1 mg/ml) or DMEM (1 ml) was added. After 15 min at 37°C, 100 ul phenylmethylsulfonylfluoride (PMSF; 80 nM) and *N*-ethylmaleimide (NEM; 200 nM) in ethanol, and 0.5 ml soybean trypsin inhibitor (SBTI; 5 mg/ml in DMEM) were added. Adherent cells were scraped loose and cells were collected by centrifugation (Sorval RT11B 15 min, 1500 rpm). Cells were lysed with 1 ml 2% (w/v) octyl glucoside in 0.01 M Tris, 0.1 M NaCl, pH 7.6 (TBS) containing 2 mM PMSF and 5 mM NEM. Lysates were collected after centrifugation (15 min, 2500 rpm) and 5 ml cold acetone was added. Precipitated proteins were dissolved in 3× Laemmli SDS solvent [8] with (reduced) or without (not reduced) 60 mM dithiothreitol (DTT). Western blots were probed with MoAb.E1, which recognizes an epitope in the C2-like domain of CAR, and analyzed as described [9].

To assess CAR synthesis and turnover following trypsin treatment, confluent cultures of HeLa cells in T25 flasks were washed two times with DMEM and covered with 1 ml DMEM. Trypsin (1 ml) was added to 0.5 mg/ml. A control sample was treated in parallel without trypsin. After 15 min at 37°C, 0.5 ml SBTI (5 mg/ml in DMEM) was added, followed by 5 ml DMEM–10% FCS. The medium was removed and centrifuged (10 min, 1200 rpm). The pelleted cells were resuspended in 5 ml DMEM–10% FCS and returned to the T25 flasks. At timed intervals, PMSF and NEM were added to the flasks and adherent cells were scraped loose, washed with TBS (containing 2 mM PMSF and 5 mM NEM) and prepared for analysis of CAR in the cell lysates. Aliquots of the final wash were used to determine the protein concentration (BCA assay, Pierce) of each sample. Equal amounts of protein from each sample were loaded on the polyacrylamide gel and CAR was detected on Western blots.

To determine whether trypsin digestion of CAR altered HeLa permissivity to CVB, HeLa cells were grown to confluence in T25 flasks. Flasks with and without trypsin treatment were inoculated with CVB3/0 [10] for 25 min, washed with medium, and returned to the 37°C incubator. Eight hours later, the cells were frozen and thawed to release virus and scraped from the flasks. Following centrifugation, supernatants were collected for titer determination and cellular material in the pellet was taken for Western blot analysis of CAR expression.

3. Results

To evaluate the susceptibility of cell surface CAR to proteolysis by trypsin, HeLa cells were grown to confluence, treated with trypsin, harvested, and lysed with octyl glucoside. Controls were processed in parallel without trypsin treatment. The immunoblots revealed that trypsin treatment produced no discernible effect on the size of CAR when analyzed without reduction (Fig. 1, lanes 1 and 2), but treatment with dithiothreitol (DTT) released an immunoreactive peptide of M_r 21 000–24 000 (Fig. 1, lane 6, arrow), with significant loss of

0014-5793/00/$20.00
PII: S0014-5793(00)02144-X

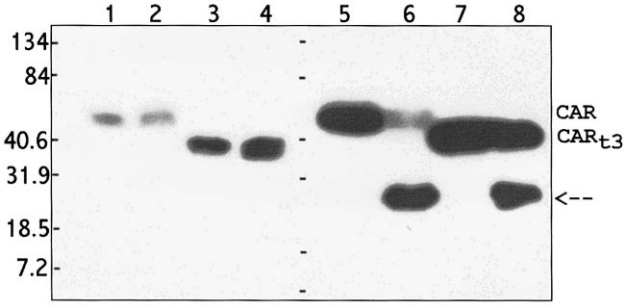

Fig. 1. CAR in HeLa (lanes 1, 2, 5 and 6) and RDt3 cells (lanes 3, 4, 7 and 8) analyzed before (odd numbered) and after (even numbered) trypsin treatment. Samples in lanes 5–8 were reduced. Numbers on the left indicate the mass (in kDa) of marker proteins corresponding to the positions marked with '-'.

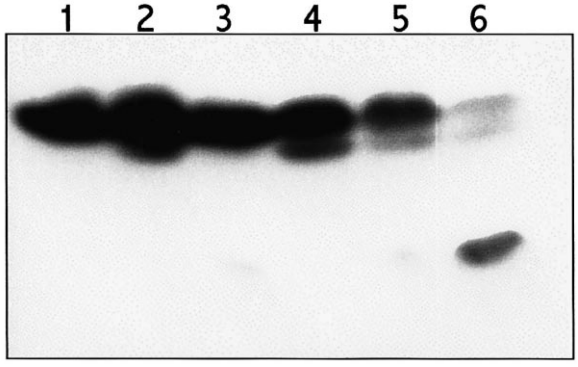

Fig. 2. CAR in confluent HeLa cells before (lane 1) or after treatment with trypsin at 0.1 mg/ml (lanes 2 and 3) or 0.33 mg/ml (lanes 4–6) for 1 min (lane 4), 5 min (lanes 2 and 5) or 15 min (lanes 3 and 6). All samples were reduced.

reactivity in the parental band. This result shows that trypsin treatment of HeLa cells results in a limited proteolysis of CAR, and the labile bonds must be located within disulfide loops such that, in the absence of reduction, no peptides are lost from the digested protein and the peptides remain cell-associated. To provide additional information concerning location of the epitope reactive with MoAb.E1 and sites cleaved by trypsin, RDt3 cells were used in similar experiments. RDt3 cells were derived from the CAR-negative RD cell line by transfection with a cytoplasmic domain-deleted cDNA derived from CAR cDNA. The construct ($HCAR_{t3}$) contains the human CAR signal sequence and residues Leu-1 through Lys-243, with an irrelevant 39-residue extension contributed by the plasmid polylinker sequence. RDt3 cells are susceptible to infection with CVB and the truncated $HCAR_{t3}$ is recognized by MoAb.E1 [9]. Trypsin treatment of the RDt3 cells produced a limited proteolysis, generating an immunoreactive peptide with the same electrophoretic mobility as that observed with trypsin-treated HeLa cells (Fig. 1, lane 8). This finding indicates that the tryptic peptide from both CAR and HCARt3 that is reactive with MoAb.E1 cannot include the cytoplasmic domain. Thus, at least one tryptic cleavage must occur within a disulfide loop of the C2-like domain. The mass of the immunoreactive tryptic peptide is consistent with a single proteolytic cleavage near the middle of the C2-like domain (between Cys-143 and Cys-193, numbered using GenBank sequence Y07593 and amino-terminal leucine [2]), or two cleavages – one near the middle of the V-like domain (between Cys-22 and Cys-101) and another in the last third of the C2-like domain (between Cys-143 and Cys-204). In other experiments, a transient band near M_r 40 000 has been detected (e.g. Fig. 2, lanes 4–6) that appears to be an intermediate product which precedes appearance of the M_r 21 000–24 000 product. This observation is consistent with the two-event model for proteolytic derivation of the M_r 21 000–24 000 product.

Further experiments determined that trypsin at 0.33 to 0.5 mg/ml was sufficient to digest most or all of the CAR expressed on confluent HeLa cells within 15 min (Fig. 2), and 15 min with trypsin at 0.5 mg/ml was used in subsequent experiments. Extended trypsin treatment diminishes the immunoreactive peptide product, indicating that additional proteolysis occurs with destruction of the epitope recognized by MoAb.E1.

The ability to detect native CAR as well as its tryptic product enabled experiments to determine the time-course of CAR replacement by new protein, as well as clearance of the digested CAR. Confluent cultures of HeLa cells were digested with trypsin (control received no trypsin) and harvested for CAR analysis at various times after replating. CAR was quantitated relative to serial dilutions of the untreated control [9], and representative blots are shown in Fig. 3. Results from multiple experiments were combined to estimate the rates at which new CAR was expressed (Fig. 4A). Loss of the 21–24 kDa peptide was quantitated relative to the maximum amount present in each of three experiments (Fig. 4B). The data in Fig. 4A indicate that CAR was replaced at an average of 3.2% ($\pm$0.4) per hour, reaching 50% of control levels about 16 h

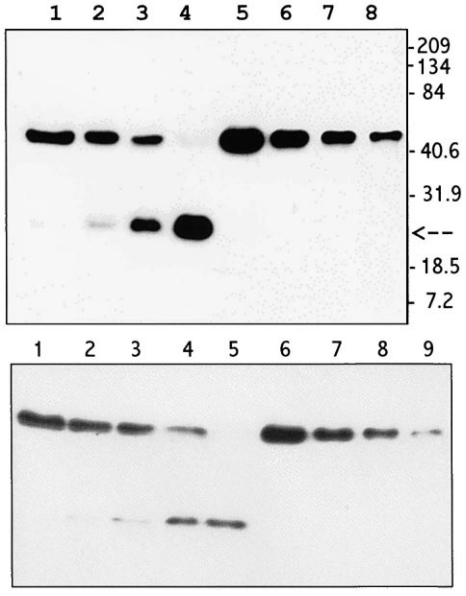

Fig. 3. CAR synthesis and turnover following trypsin treatment. Two experiments are shown. In the upper panel, samples 5–8 correspond to the untreated control (5) and two-fold serial dilutions (6–8). Lanes 1–4 contained samples prepared 20.7, 12, 5.6, and 0 h after trypsin treatment, respectively. The arrow points to the 21–24 kDa tryptic peptide recognized by MoAb.E1. In the lower panel, samples 6–9 correspond to the untreated control (6) and serial two-fold dilutions to provide a standard curve for quantitative analysis (7–9). Lanes 1–5 contained samples prepared 24.3, 13, 9.2, 3.9, and 0 h after trypsin treatment, respectively.

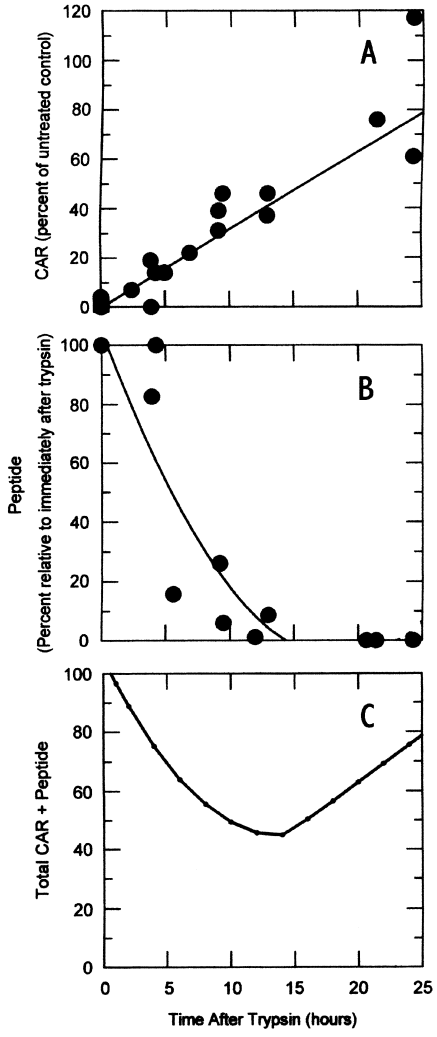

Fig. 4. Synthesis of new CAR (A) and removal of trypsin-digested CAR (B) from HeLa cells after treatment with trypsin. Panel C shows the results of combining the curves in panels A and B.

after trypsin treatment. The four experiments shown in panel A gave very similar results, while a single experiment (not shown) gave a slower rate of recovery. Removal of proteolytically cleaved CAR (detected as the 21–24 kDa proteolytic peptide) was not linear with time, and the peptide was 50% removed by about 6 h following trypsin treatment. These results show that the cleaved receptor is removed from the cell more rapidly than new CAR is expressed (Fig. 4A and B). The different rates at which cleaved but functional receptor is cleared and replaced by newly synthesized receptor should produce a window during which minimum levels of receptor are present, and this minimum can be predicted to occur several hours after trypsin treatment. Fig. 4C, derived by combining the curves in Fig. 4A and B, shows that if the cleaved receptor is equivalent to 100% of CAR immediately following trypsin treatment, receptor levels will decrease for the ensuing 14 h to a nadir near 40% of control levels. This nadir will be followed by recovery, reaching 100% of control levels only after an additional 14 h or more. If the cleaved but functional CAR immediately after trypsin treatment corresponds to only 50% of the CAR present before trypsin treatment, the nadir in receptor expression would occur at about 8 h post-trypsin.

To determine whether trypsin digestion of CAR altered HeLa permissivity to CVB, HeLa cells with and without trypsin treatment were inoculated with CVB3. Eight hours later, CAR remained readily detectable in HeLa cells infected with CVB3/0 in the absence of trypsin treatment (Fig. 5, lane 1), which is consistent with the report that CVB infection does not reduce CAR expression in endothelial cells [9]. In contrast, CAR remained markedly reduced in HeLa cells infection with CVB3 after treatment with trypsin (Fig. 5, lane 2), indicating that synthesis of new host protein, which is blocked soon after CVB infection, is required to re-express intact CAR. A band near 40 kDa is pronounced in the sample that was infected without trypsin treatment (Fig. 5, lane 1) suggesting that proteolysis or some other modification of CAR occurs during the infection. HeLa cells infected after trypsin treatment produced CVB3 (1.58×10^8 $TCID_{50}$) equivalent to that obtained from HeLa cells that were not trypsin-treated ($1.58–2.11\times10^8$ $TCID_{50}$), indicating that the infection after limited proteolysis of CAR proceeds with efficiency comparable to infection of cells bearing intact CAR.

4. Discussion

More than 30 years ago, Crowell and colleagues [5–7] reported that CVB3 binding to HeLa cells was unaffected by trypsin treatment. Experiments reported here reveal that the CAR is cleaved within 15 min following addition of trypsin to HeLa cells. The limited proteolytic cleavage leaves CAR associated with the cell and the peptides remain associated through disulfide bonds. This altered protein appears to fully support infection by CVB3. In contrast to the earlier work showing that virus binding after treatment of HeLa cells with chymotrypsin was 50% restored in about 4 h, the current experiments found that 50% restoration of intact CAR protein following trypsin treatment required about 16 h. This difference may be due to the binding of CVB3 by multiple proteins on the cell surface (e.g. DAF and CAR), whereas the current experiments specifically measured the CAR protein. It is also noted that the different rates were determined after treatment with different proteases which could potentially in-

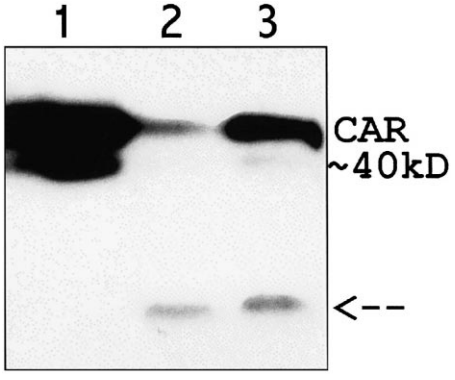

Fig. 5. CAR in HeLa cells after 8 h infection with CVB3 with and without trypsin treatment prior to inoculation. Lane 1: HeLa cells infected with CVB3 without trypsin treatment. Lane 2: HeLa cells treated with trypsin and inoculated with CVB3. Lane 3: HeLa cells treated with trypsin and not infected.

fluence the rate at which CAR is synthesized. Irrespective of these differences, full recovery of CVB3 binding after treatment with chymotrypsin as well as expression of newly synthesized CAR in trypsin-treated HeLa cells requires more than 24 h after removal of the protease. Pertinent to contemporary studies of CAR and CVB infection, these observations suggest that polarized epithelial cells in which CAR is not accessible to virus [11] should be readily infectable after trypsinization, even though the CAR will be altered. Furthermore, results from the kinetic analyses of CAR expression after proteolysis caution that studies that may be influenced by receptor density or by residual proteolytically altered CAR should be conducted at least 24 h after passaging cells using trypsin.

Acknowledgements: I thank Drs. Steven Tracy and Nora Chapman for their collegial interest and suggestions. Mr. Justin Hobbs provided technical assistance with these studies.

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