Serum-Free Transient Protein Production System Based on Adenoviral Vector and PER.C6 Technology: High Yield and Preserved Bioactivity

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ABSTRACT: Stable E1 transformed cells, like PER.C6, are able to grow at scale and to high cell densities. E1-deleted adenoviruses replicate to high titer in PER.C6 cells whereas subsequent deletion of E2A from the vector results in the absence of replication in PER.C6 cells and drastically lowers the expression of adenovirus proteins in such cells. We therefore considered the use of an ΔE1/ΔE2 type 5 vector (Ad5) to deliver genes to PER.C6 cells growing in suspension with the aim to achieve high protein yield. To evaluate the utility of this system we constructed ΔE1/ΔE2 vectors carrying different classes of protein, that is, the gene coding for spike protein derived from the Coronavirus causing severe acute respiratory syndrome (SARS-CoV), a gene coding for the SARS-CoV receptor or the genes coding for an antibody shown to bind and neutralize SARS-CoV (SARS-AB). The ΔE1/ΔE2A-vector backbones were rescued on a PER.C6 cell line engineered to constitutively over express the Ad5 E2A protein. Exposure of PER.C6 cells to low amounts (30 vp/cell) of ΔE1/ΔE2 vectors resulted in highly efficient (>80%) transduction of PER.C6 cells growing in suspension. The efficient cell transduction resulted in high protein yield (up to 60 picogram/cell/day) in a 4 day batch production protocol. FACS and ELISA assays demonstrated the biological activity of the transiently produced proteins. We therefore conclude that ΔE1/ΔE2 vectors in combination with the PER.C6 technology may provide a viable answer to the increasing demand for high quality, high yield recombinant protein.

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KEYWORDS: protein production; human PER.C6® cells; recombinant adenovirus; serum-free medium

Introduction

To date different platforms are used for production of recombinant proteins ranging from bacteria, yeast and fungi to insect-, plant-, and mammalian cells (Andersson et al., 2001; Bao and Fukuhara, 2001; Boedeker, 2001; Cai, 2001; Chen et al., 2001; Friehs and Reardon, 1993; James and Lee, 2001; Jenson and Johnsen, 2001; Konishi et al., 2002; Lazaris et al., 2002; Levy et al., 2001; Mattes, 2001; Massaer et al., 2001; McQuillan et al., 2001; Menassa et al., 2004; Sarkar and Sen, 1998). Given the importance of human type post-translational and peri-translational processing for protein half-life, biological activity, secretion, and protein yields (Berg et al., 1993; Breitbach and Jarvis, 2001; Fussenegger et al., 1999; Jenkins et al., 1998; Kawashima et al., 1993; Lawrence et al., 2001; Lee et al., 1989; Marchal et al., 2001; Mosser et al., 1994; Seo et al., 2001), either human derived cell lines or non-human systems adapted to express human enzymes involved in post-transcriptional modification of proteins, are preferred (Côté et al., 1998; Lawrence et al., 2001; Marchal et al., 2001; Seo et al., 2001). However, mammalian cell-based protein production is time-consuming since it requires stable cell line generation, sub-cloning, screening, and cell clone-selection. Moreover, this process is costly due to the required safety testing of both candidate products as well as cell banks. As such this process does not allow for the rapid production of high quality recombinant protein batches required for candidate protein selection or rapid evaluation of a candidate protein in a clinical setting. Because of these limitations, research attention focuses on novel systems and technology for transient production of high quality protein. Although predominantly large-scale DNA transfection is being investigated (Baldi et al., 2007; Derozzi et al., 2004; Tait et al., 2004) adenoviral vectors are being considered as well (Andersen and Krummen, 2002; Côté et al., 1998; Garnier et al., 1994; Gossen et al., 1994; Massie et al., 1995; Sarkar et al., 2001; Tom et al., 1995; Wu et al., 1996). Attractive features of the adenoviral vector approach for transient protein production include (i) the ability of adenovirus to actively bind to cells growing in suspension, (ii) the high efficiency of vector internalization, and (iii) the high efficiency of nuclear transport of the...
Materials and Methods

Generation of PER.E2A Cell Line and Cell Culture

The complete ts125E2A-coding region was PCR-amplified from DNA isolated from the temperature sensitive adenovirus mutant H5ts125 (Ensinger et al., 1972; Vliet and Sussenbach, 1975; Vliet et al., 1975) with primers DBPpcr1 and DBPpcr2 using the ExpandTM Long Template PCR system according to the standard protocol of the supplier (Boeringer, Mannheim, Germany). The primer DBPpcr1: CGG GAT CCC CCA CC A TG TGG CCA GTC GGG AAG AGG AG (5′–3′) contained a unique BamH I restriction site (underlined) 5′ of the Kozak sequence (italic) and start codon of the E2A coding sequence (bold). The primer DBPpcr2: CGG AAT TCT TAA AAA TCA AAG GGG TTC TGG CGC (5′–3′) contained a unique EcoRI restriction site (underlined) 3′ and the stop codon of the E2A coding sequence (bold). The PCR fragment was digested with BamH I/EcoRI and cloned into plasmid pcDNA3 (Invitrogen, Breda, The Netherlands) and sequenced giving rise to pcDNA3tsE2A. For transfer of the E2A region, PER.C6 cells were transfected with 5 µg pcDNA3 plasmid containing no insert, wild type E2A, or ts125E2A using the lipofectAMINE Plus reagent kit according to the protocol supplied (Invitrogen). Seventy-two hours after transfection, cells were seeded in DMEM/10% FBS/10 mM MgCl2, supplemented with 0.25 mg/mL G418 to select for transfectants. Stable transfected clones were routinely maintained in the same medium, however the G418 selection was withdrawn from the cultures at the moment ΔE1/ΔE2 viruses were produced. The E2A expression levels in different cell clones were determined by Western blotting using monoclonal antibody B6 (Reich et al., 1983) and horseradish-peroxidase-conjugated-goat anti mouse antibody (BioRad, Hercules, CA) as first and second antibody, respectively. Western blotting procedure and incubations were performed according to the protocol provided by Millipore (Billerica, MA). The complexes were visualized with the ECL detection system according to the manufacturer’s protocol (Amersham, Piscataway, NJ). In addition, the selected cell lines were tested for their ability to complement the deleted E2A gene function of adenovirus Ad5.d1802 (Rice and Klessig, 1985) by demonstrating the appearance of cytopathic effect (CPE) upon infection at 32°C. To obtain cells growing in suspension, one selected PER.E2A cell line was cultured at 39°C and 10% CO2 in a 175 cm2 tissue culture flask in DMEM, supplemented with 10% FBS and 10 mM MgCl2. At 70–80% confluency cells were washed with PBS (NPBI) and the medium was replaced by 25 mL serum-free suspension medium SFM consisting of Ex-cellTM 525 (JRH) supplemented with 1× l-glutamine (Gibco BRL). Two days later, cells were detached from the flask by flicking and the cells were centrifuged at 1,000 rpm for 5 min. The cell pellet was re-suspended in 5 mL SFM and 0.5 mL cell suspension was transferred to an 80 cm2 tissue culture flask, together with 12 mL fresh SFM. After 2 days, cells were harvested. Next cells were seeded in a 125 mL tissue culture Erlenmeyer (Corning, Corning, NY) at a seeding density of 3 × 105 cells/mL in a total volume of 20 mL SFM. Cells were further cultured at 125 rpm on an orbital shaker at 39°C, 10% CO2. Cells were counted at days 1–6 in a Burker cell counter. Human PER.C6 cells are routinely maintained in a humidified 37°C environment in Dulbecco’s Modified Eagle medium (DMEM) supplemented with 10 mM MgCl2, either in the presence or absence of fetal bovine serum (FBS). Human A549-cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained in DMEM/10% FBS. For infection, PER.C6 cells were seeded in roller bottles (100 mL volume) at 0.3 × 10⁶ cells/mL in AEM medium (Invitrogen) supplemented with l-glutamine. Cells were cultured in roller bottles (2 rpm) in a 37°C incubator at 10% CO2. When cell concentrations reached 2 × 10⁶ cells/mL, cultures were centrifuged for 10 min at 500g and pellets were re-suspended in 100 mL fresh AEM/l-glutamine at a final cell concentration of 2.5 × 10⁶ cells/mL. The next day cells were counted using a Casey counter (Scharfe system).
and virus was added to the roller bottles corresponding to the desired MOI. For infection of PER.C6 cells in shaker flasks (25 mL culture volume) cells cultured in roller bottles were counted, centrifuged and cell pellets were re-suspended in fresh AEM/l-glutamine at 2.5 x 10^6 cells/mL after which virus was added at the desired MOI. Cells were cultured in shaker flasks at 75 rpm, 37°C, 10% CO₂. Harvest of medium was done between day 3 and 14, depending on the type of experiment and the protein expressed.

**Construction of ΔE1/ΔE2 Plasmid System and Vector Generation**

Recombinant ΔE1/ΔE2 viruses were generated by a homologous recombination procedure using an identical plasmid-based system as previously described for the production of E1-deleted vectors (Havenga et al., 2001) with the exception that a modified cosmid (pWE/Ad.AflII-rITR.DE2A) lacking the E2A gene was used. To remove the E2A from the adenoviral backbone assembly PCR amplification and standard molecular cloning techniques were used. Co-transfection into mammalian cells of this cosmid with an expression plasmid encoding the left 5 kb of the adenoviral genome (pAdapt), results in homologous recombination leading to a complete Ad5 genome. The cDNA (3,767 bp) coding for full-length SARS-CoV S1-spike (Brink et al., 2005) was cloned into the expression cassette of pAdapt whereas also the 1,695 bp fragment of S1-spike obtained through restriction analyses was cloned into the expression cassette of pAdapt in order to obtain a ‘secreted’ or ‘soluble’ form of S1-spike (pAdapt.S565). Likewise, the 2,417 bp fragment coding for complete ACE2 (Genbank sequence AF291820) and a truncated version (sACE2) lacking the trans-membrane region (aa residues 741–805 of ACE2) were cloned into pAdapt. Both soluble proteins (S565 and sACE2) were fused in frame and upstream of a Myc-His tag to enable simple purification and detection of these expressed proteins. To create a ΔE1/ΔE2 vector carrying the light chain (LC) and heavy chain (HC) coding for a SARS-CoV neutralizing antibody (Meulen et al., 2004) the LC and HC genes were subcloned into plasmids that already contained appropriate leader sequences and either the constant CH1, CH2, and CH3 regions of the HC or C kappa, the constant region of the LC. To enable translation of HC and LC from one transcript, a Internal Ribosome Entry Site sequence derived from the Encephalomyocarditis Virus (Jackson et al., 1990) was inserted in between HC and LC and cloned in the expression cassette of pAdapt. Generation of recombinant ΔE1/ΔE2 adenovirus batches was accomplished at 32°C on adherent PER.E2A cells using Lipofectamine2000 transfection of pAdapt carrying various transgenes and cosmid (pWE/AflII-rITRDE2A) according to instructions provided by the manufacturer (Invitrogen). Homologous recombination between sequences shared between pAdapt and pWE/AflII-rITRDE2A resulted in formation of ΔE1/ΔE2 vector visible by the onset and progression of CPE. Purified virus stocks were obtained using standard two-step CsCl-gradient banding and the isolated virus was dialyzed in three steps to a final formulation in PBS/5% (w/v) sucrose. Virus concentrations were determined based on optical density method (Maizel et al., 1968) in the presence of 1% (w/v) SDS. Infectivity was measured by TCID₅₀ assay on PER.E2A cells (3 weeks post-infection) as described previously (Fallaux et al., 1996).

**Protein and DNA analyses**

Detection of adenoviral proteins in infected cells and DBP protein in PER.E2A cells was performed by Western blot analysis. Hereto, 20 μg of total cellular protein was loaded on a polyacrylamide gel. To visualize DBP, fiber, penton, the pTP/TP proteins, or the E4-orf6 protein, antibodies B6-mouse (A kind gift from N. Reich, New York, USA; Reich et al., 1983), E641/3-rabbit (a kind gift from R. Gerard, Leuven, Belgium), Pb571-rabbit (a kind gift from P. Boulanger, Montpellier, France), pTP-396-rabbit (a kind gift from P. van Vliet, Utrecht, the Netherlands), RSA-3-mouse (a kind gift from P. Branton, Montreal, Canada), were used at dilutions of 1/1,000, 1/1,000, 1/1,000, 1/750, and 1/377, respectively. Primary antibody binding was visualized utilizing a second antibody (ECL detection kit), that is Goat-anti-mouse, IgG, H + L, (BioRad) or Goat-anti-rabbit, IgG H + L, both at a dilution of 1/7,500. For Southern blot analyses, viral DNA was isolated from infected cells as described previously (Zhang et al., 1995). Viral DNA was digested with EcoRI, liberating the LacZ coding domain from the adenoviral backbone. Blots were probed with a ³²P-labeled EcoRI fragment of the LacZ cDNA. Purified S565; sACE2 and SARS-AB were fractionated by SDS-PAGE on 10% Bis-Tris gels and ran in MOPS running buffer (Invitrogen) and stained using Colloidal blue staining (Invitrogen) according to the supplier protocol. For Western blot analysis, fractionated proteins were transferred onto Immobilon-P membranes (Millipore) and incubated with (1:10,000) HRP-conjugated donkey-a-human Mab (Jackson ImmunoResearch, Suffolk, UK) in case of SARS-AB and with anti-c-Myc-HRP (1:10,000) for both S565 and sACE2. Efficiency of gene transfer was typically assessed 48 h after virus addition by determination of LacZ transgene expression either by staining the intact cells as described previously (Havenga et al., 2001) or by preparing a cell lysate of the cells and measuring β-galactosidase activity in the total cell lysate using a β-galactosidase activity kit (Promega, Madison WI). Transfection experiments were performed with 5 μg of plasmid DNA purified via Qiagen columns (Westburg, The Netherlands). Both soluble (Myc-His tagged) proteins S565 and sACE2 as well as the SARS-AB were produced in 100 mL roller bottles, medium was collected and centrifuged at 500g and filtered using a 0.22 μm bottle top filter. S565 and sACE2 proteins were purified on an AKTA explorer (GE Healthcare, Brussels, Belgium) using HisTrap HP (1 mL) affinity and HiTrap desalting (5 mL) columns (GE Healthcare) using the
appropriate buffers as recommended by the supplier. Total protein concentration in culture harvests was determined using the Micro BCA™ protein assay (Pierce; The Netherlands) according to instructions provided by the manufacturer. Samples of the harvests were also serial diluted and separated on SDS–PAGE gels, after colloidal blue staining the gel was optically scanned and all single products (in the linear sample range) present in the individual lanes were digitally integrated and converted to integrated optical density (IOD) values using Gel-Pro analyzer developed by Media Cybernetics, Inc. (Bethesda, MD). This enabled the expression of the individual proteins as percentage of total protein per lane and is defined as protein purity. Subsequently, the amount of produced S655 and sACE2 was calculated by multiplication of the purity by the total amount of protein. The SARS virus neutralizing antibody, present in culture medium was purified by HPLC using HiTrap rProtein A protA and HiPrep 26/10 affinity and desalting columns (GE Healthcare) and quantified by optical density measurements at 280 nm. Detection of ACE2 on PER.C6 cells was performed by exposure of PER.C6 cells to a ΔE1/ΔE2 vector carrying the ACE2 coding domain. After 48 h, transiently expressed ACE2 on PER.C6 cell membranes was detected by FACS analysis after cell staining with goat-anti human ACE2 ectodomain polyclonal (0.4 μg/mL; R&D Systems, Oxford, UK) and PE-conjugated donkey anti-Human IgG (Jackson ImmunoResearch). For the detection of proper binding of sACE2 to S1-spike, PER.C6 cells were infected with a ΔE1/ΔE2 vector carrying the S1-spike and after 48 h cells were incubated with transiently produced and purified sACE2 (40 μg/mL) and subsequently with mouse-anti- c-Myc (9E10) FITC (6 μg/mL; Santa Cruz Biotechnologies, Inc., Santa Cruz, Calif.). All incubations and washes were performed at 4°C in PBS supplemented with 1% BSA. Cells were analyzed on a FACSCalibur with CELLQuest Pro software (Becton Dickinson, Franklin Lakes, NJ). To determine functionality of the SARS virus neutralizing antibody, microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with anti-Myc antibody (5 μg/mL PBS, pH 7.4) followed by incubation with purified Myc-tagged S655 (2 μg/well). Concentrations of a transiently or stably produced SARS virus neutralizing antibody range (0.18–370 ng/mL) were added and incubated with (2,000 times diluted) PE-conjugated donkey anti-Human IgG (H+L; Jackson ImmunoResearch). The assay was further developed using 4-phenylenediamine substrate and stopped by the addition of 1 M H₂SO₄. The absorbance was measured at 492 nm.

Results

Generation of the PER.E2A Cell Line and Production of ΔE1/ΔE2A Vector

Transfection in PER.C6 cells of a plasmid containing a Neomycin resistance (G418R) gene and the E2A coding domain (Vliet and Sussenbach, 1975) under the regulation of the CMV-promoter, resulted in >100 G418R colonies from which 45 clones were further tested resulting in 37 established cell lines. Each cell line produced high levels of DNA binding protein (DBP) driven from the E2A gene as determined by Western analysis (data not shown). From the 37 cell lines the PER.E2A cell line was selected based on the capability of the cells to stably express the E2A gene in the absence of G418 and the ability to grow in suspension in the absence of animal serum. The PER.E2A cell line was compared to PER.C6 cells showing that during a 10 days culture period at 39°C the proliferation kinetics of the two cell lines were identical (data not shown). At 32°C, both cell lines behaved identical until after approximately 5 days in culture when the cell viability of the PER.E2A cell line started to decrease as a result of E2A toxicity (data not shown) thus providing a 5-day time frame for virus replication and progeny formation of ΔE1/ΔE2A adenoviral vectors. Next, the efficiency of ΔE1/ΔE2A recombinant vector generation on PER.E2A cells in relation to cell culture temperature was established. Hereto, an ΔE1/ΔE2A vector and as control an ΔE1 virus, both carrying the LacZ reporter gene, were generated on PER.E2A and PER.C6 respectively and purified. These vector stocks were used to transduce both PER.C6 and PER.E2A cells at a concentration of 100 virus particles per cell after which cells were cultured at different temperatures. Forty-eight hours later, the amount of infectious virus particles formed was determined by exposing human A549 indicator cells to 100 μL of cleared supernatant and subsequently counting the number of LacZ positive cells obtained. As shown in Figure 1A, an ΔE1 vector can be produced both on PER.C6 as well as PER.E2A cells whereas an ΔE1/ΔE2A vector can be produced only on PER.E2A cells. Moreover, a temperature of 34°C proved optimal for production of an ΔE1/ΔE2A vector on PER.E2A. A subsequent time-course experiment whereby supernatant was harvested at 24, 48, 72, or 120 h after virus exposure demonstrated that the highest titer of ΔE1/ΔE2A vector could be obtained at 72 h post-virus exposure (data not shown).

Using these parameters, more than 25 batches of recombinant ΔE1/ΔE2A viruses carrying different transgenes have since been produced and purified on PER.E2A cells with averaged yield of 4.7 × 10¹¹ virus articles per milliliter and a virus particle per infectious unit ratio (VP/IU) of 22.4 ± 17.9 (average ± SD). These production data are similar to data as established for ΔE1 adenoviral vectors production on PER.C6 cells. A sensitive PCR analyses, discriminating between E2A plus and E2A minus virus, performed on purified virus batches indicated that reversion, that is homologous recombination between E2A DNA in the cell and the viral genome, possibly leading to E2A virus does not occur (data not shown).

Replication Specificity and Viral Gene Expression of an ΔE1/ΔE2A Vector

To investigate the replication potential of an ΔE1/ΔE2A and as control ΔE1 recombinant vector in PER.C6 and PER.E2A
in relation to cell culture temperature, both cell types were cultured at different temperatures and subsequently transduced with either a ΔE1 or an ΔE1/ΔE2A vector. Forty-eight hours after vector exposure, viral DNA was isolated and Southern analysis was performed. From the results, shown in Figure 1B, the following conclusions were drawn: (i) at 32, 34, and 37°C both an ΔE1 and ΔE1/ΔE2A vector can efficiently replicate in PER.E2A cells resulting in the clear detection of viral DNA copies at 48 post-virus exposure, (ii) at 39°C the replication efficiency of an ΔE1/ΔE2A vector is significantly reduced due to the absence of functional E2A protein, and (iii) an ΔE1/ΔE2A vector exposure to PER.C6 cells did not result in detection of viral copies indicative for the absence of viral replication. Since E2A is indirectly involved in activation of the viral major late promoter (MLP), responsible for driving late gene expression (structural proteins), deletion of this region from the adenoviral genome should result in absence of viral capsid proteins in transduced cells. To investigate capsid protein expression, human A549 indicator cells were exposed to an increasing concentration of ΔE1 or ΔE1/ΔE2A vector and Western analyses on protein lysates was performed. As shown in Figure 1C, even at 10,000 virus particles per cell no expression could be observed from either penton or fiber capsid at 48 h after ΔE1/ΔE2A vector exposure. In contrast, expression of both penton and fiber capsid proteins could be detected after exposure of 1,000 virus particles per cell using a ΔE1 vector. Expression of pre-terminal protein (pTP) and terminal protein (TP) derived from the viral E2B region, involved exclusively in promoting viral DNA replication in the PER.E2A cell line, as well as E4 derived orf6 protein (E4) involved in trans-activation of the CMV promoter driving expression of the gene encoding for the protein of interest (Gorziglia et al., 1999; Lusky et al., 1998) could still be detected. Finally, we determined whether equal levels of expression from an inserted transgene could be obtained using an ΔE1/ΔE2A as compared to a ΔE1 recombinant virus. Hereto, human A549 indicator cells were transduced with equal doses of vector carrying the LacZ marker gene. Since levels of reporter gene expression proved not
significantly different using either LacZ (Fig. 1D) it was concluded that the vectors are comparable with regard to transduction capacity, capsid stability, and CMV-mediated protein expression. Based on the data obtained thus far it was concluded that an ΔE1/ΔE2A adenoviral vector can be efficiently produced at 34°C on PER.C6 cells and can subsequently serve as a tranducing agent for PER.C6 cells without inducing viral backbone replication or high level viral capsid protein expression in human A549 indicator cells.

**ΔE1/ΔE2A Vector Mediated Protein Production in PER.C6**

Taking advantage of the observation that ΔE1/ΔE2 vectors do not replicate on PER.C6 cells we were interested to investigate if recombinant protein could be efficiently expressed in PER.C6 cells using this vector. To investigate yield, five ΔE1/ΔE2 vectors were generated and purified, one carrying the SARS-CoV soluble spike protein (S565), one carrying the complete SARS-CoA Spike protein (S1-spike), one carrying the soluble SARS virus receptor (sACE2), one carrying the complete ACE2 cellular receptor, and one carrying the heavy and LC coding for an SARS virus neutralizing antibody (ter Meulen et al., 2004). These proteins were chosen since they represent different classes of proteins and to determine bioactivity of the proteins in binding assays (SARS-AB binds S1-spike and both S1-spike and S565 bind to the ACE2 receptor). The ΔE1/ΔE2 vector coding for complete ACE2 protein was subsequently used to transduce PER.C6 suspension cells cultured in 100 mL roller bottles at increasing concentrations of virus particles per cell. Staining of the cells, 48 h after vector exposure, with a commercially available anti-ACE2 antibody demonstrated that approximately 90% of cells growing in suspension clearly produced the ACE2 protein using as little as 30 virus particles per cell as inoculation dose (Fig. 2A). In a subsequent experiment, shaker flasks (25 mL, 2.5 × 10⁶ cells/mL) were inoculated with 10, 30, or 100 virus particles per cell using the ΔE1/ΔE2 vector expressing the SARS-AB. As shown in Figure 2B the antibody yield increased with increasing inoculation dose up to 60 μg/mL (at 100 VP/cell) over an 8-day batch production protocol. Although the highest antibody concentration was obtained with the highest virus particle per cell concentration, 30 virus particles per cell was chosen as inoculation dose for the remainder of the experiments. Experiments performed thus far used caesium chloride purified ΔE1/ΔE2 vectors (Fang et al., 1996; Goldman et al., 1995; O’Neal et al., 1998). Clearly, timelines required to produce protein could be significantly reduced if PER.C6 cells could be inoculated with non-purified vector stocks produced on the PER.2A cell line. Therefore a head-to-head comparison was performed whereby four infectious units per cell of either non-purified (cleared vector stock) or purified vector stock (caesium chloride gradient purified) was used to inoculate PER.C6 cells. As shown in Figure 2C the yield of SARS anti-body produced did not significantly differ demonstrating that cleared vector stock directly harvested from he PER.2A cell line can be used for production of recombinant protein without compromising yield.

Next, roller bottles containing 2.5 × 10⁶ PER.C6 cells/mL (100 mL) growing in suspension in absence of serum were inoculated with 30 virus particles per cell using the ΔE1/ΔE2 vector carrying the sACE2 receptor. Four days post-inoculation cells were harvested demonstrating that the total cell count had not increased nor decreased over the 4-day vector exposure period. A volumetric productivity of 611 μg/mL and specific productivity of 61 pg/cell/day (611/4 days/2.5 million cells) was obtained using this production protocol. Inoculation of roller bottles with 30 virus particles per cell of the ΔE1/ΔE2 vector coding for S565 resulted in a volumetric productivity of 370 μg/mL or specific productivity of 37 pg/cell/day. The SARS antibody yielded 140 μg/mL or specific productivity of 7 pg/cell/day during a 8 day batch production protocol.

Since the sACE2 and S565 were generated carrying a Myc-His tag they could be easily purified using a commercially available (Nickel-based) HisTrap HP column that has affinity for the included histidine residues, whereas the SARS antibody was purified using HPLC as described in the Material and Method Section. Purified proteins were run on SDS–PAGE gel and visualized either by whole protein staining (Collodial blue staining) or Western Blot analyses (Fig. 3 panels A and B, respectively) demonstrating protein integrity of transiently produced proteins without apparent aberrant protein being formed.

To assess the bioactivity of the produced proteins two sets of experiments were performed. In the first set of experiments PER.C6 cells transiently expressing complete S1-spike protein were incubated with purified sACE2 protein and subsequently stained with anti-c-Myc (9E10) FITC labeled antibody. As shown in Figure 4A almost all cells expressed on their membrane the S1-spike protein resulting in clear binding of the sACE2 protein as witnessed by high mean fluorescence upon staining with the anti-c-Myc antibody. In the second set of experiments, plates were coated with anti-Myc to capture S565 protein, which in turn was used to capture the SARS-AB. The ELISA was performed using an antibody dose titration and head-to-head comparison of the transiently produced antibody versus the SARS-AB produced and purified from PER.C6 cells that stably expresses this antibody (specific activity 14 pg/cell/day). As shown in Figure 4B no significant difference in binding affinity of the antibody could be observed using transient produced antibody as compared to the antibody produced from a stable cell line.

Collectively the data obtained demonstrate that (i) ΔE1/ΔE2 vectors are suitable for the production of different classes of proteins, that is, viral protein, cellular protein or antibody, (ii) an inoculation dose of 30 virus particles per cell suffices for significant protein yield without the need to purify vector inoculation stocks and is eligible for further optimization, and (iii) proteins produced and purified
demonstrate proper integrity and bioactivity as witnessed in binding assays and compared to protein produced in stable cell lines. As such, this platform could deliver high yield recombinant protein batches within approximately 30 working days (see projection in Fig. 5) although clearly further optimization is currently required to achieve vector generation and titration within approximately 4 weeks in order to perform large scale protein production.

**Discussion**

Several reports have previously described the use of a replication deficient adenovirus in combination with adenovirus production cells (HEK293) with the aim to produce recombinant protein (Andersen and Krummen, 2002; Côté et al., 1998; Garnier et al., 1994; Lamarche et al., 1990; Massie et al., 1995, 1998). In these studies, the E1-deleted vector was allowed to replicate in HEK293 cells, in the process yielding high amounts (4–20% total cell protein) of the protein of interest. However, besides the formation of replication incompetent vectors during production it has been demonstrated that homologous recombination can occur in HEK293 cells between vector and cell line sequences resulting in the formation of RCA (Imler et al., 1995; Lochmüller et al., 1994). Another disadvantage of the reported systems is the relative short time frame allowed for protein production given the fact that the adenovirus lyses the cells that produce the protein of interest within days.

To improve upon the existing systems, PER.C6 cells were chosen as a starting point for the generation of a transient protein production platform (Fallaux et al., 1998). This cell line has been extensively documented, cell banks meet all pertinent US and EEC regulatory requirements, cells can be cultured in suspension without the aid of micro-carriers to high cell densities (>10^7 cells/mL), cells can be cultured in suspension in the absence of any serum components, and cultures can be scaled to large bioreactors. In addition, the formation of RCA vectors has never been reported. To meet other criteria, that is absence of replication deficient virus particles and de novo synthesis of viral capsid proteins that could negatively influence cell viability and proliferation, an adenovirus deleted in its genome for both the E1/E2A regions was generated. Deletion of these regions has been previously shown to result in absence of almost all viral gene expression (Gorziglia et al., 1999; Lusky et al., 1998; O’Neal et al., 1998).
et al., 1998) without compromising CMV promoter activity driving foreign gene expression (Gorziglia et al., 1999; Lusky et al., 1998). This report demonstrates that a ΔE1/ΔE2A vector is unable to replicate in PER.C6 cells and does not express viral capsid proteins even after high dose inoculation of susceptible human A549 cells.

We therefore postulated that this vector could provide a safe reagent to introduce DNA into PER.C6 cells growing in suspension for scalable transient protein production purposes. In order to produce high-titer ΔE1/ΔE2A vector we established the PER.E2A cell line. Hereto, a temperature sensitive mutant E2A gene (Vliet and Sussenbach, 1975) was employed since wild type E2A protein expression is known to be toxic to cells (Klessig et al., 1984). The temperature sensitive mutant carries a mutation in codon 413 of the DNA binding protein transcribed from the E2A region of adenovirus serotype 5 (Kruijer et al., 1981), which results in functional E2A protein at 32°C but not at 39°C. This allowed the establishment of a stable E2A expressing cell line that could be cultured without apparent growth deficit or viability loss at 39°C as compared to parental PER.C6 cells. For production of the ΔE1/ΔE2 vector the PER.E2A cells were switched to a temperature of 32°C, which allowed a 5 day time window for vector production before cell viability started to decrease due to E2A expression. To ensure scalability of the platform the PER.E2A cells were adapted to grow in suspension, in the absence of serum components.

This report thus describes for the first time a transient protein production system that can be as safe as the use of naked DNA for large scale transient protein production but solves the current technological bottlenecks associated with

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**Figure 3.** PER.C6 cells cultured in suspension in absence of serum in rollerbottles were inoculated with 30 vp/cell of a ΔE1/ΔE2 vector carrying the S565 coding domain, the ACE2 coding domain or the SARS-AB coding domain. Transiently produced and purified proteins (S) were separated on SDS–PAGE gels (panel A) and analyzed via Western blot analyses (panel B). S565 and sACE2 were visualized with mouse-anti-c-myc (HRP-conjugated) monoclonal antibody. SARS-AB was visualized with donkey-anti-human IgG (HRP-conjugated) monoclonal antibody. The molecular weight marker is represented (M). Purified S565 transiently produced in HEK293 cells (P), and stably produced CR3014 on PER.C6 cells (C) were included as positive controls. The contents of various lanes are not standardized for equal protein amounts.

**Figure 4.** A: PER.C6 cells were exposed to 30 vp/cell of an ΔE1/ΔE2 vector carrying the S1-spike coding domain. Forty-eight hours post-exposure cells were harvested and stained with 40 μg/mL transiently produced sACE2. To visualize sACE2 binding to S1-spike cells were further incubated with mouse-anti-c-myc-FITC. Unstained infected PER.C6 cells were used to set back ground cell staining at 1%. B: The SARS virus neutralizing antibody produced transiently using a ΔE1/ΔE2 vector (triangles) was compared to the identical antibody produced on PER.C6 cells stably expressing this antibody (diamonds) via ELISA. Binding of the SARS-AB to S565 in a dose dependent manner was detected using peroxidase-conjugated AffiniPure donkey anti-human IgG (Jackson Immuno Research).
the use of naked DNA transfection, that is, limited internalization of plasmid DNA into mammalian cells and limited transport of plasmid DNA to the nucleus. Several improvements to this system that will be investigated next include the set-up of a fast and reliable RT-PCR or FACS-based virus titration assay, needed to replace the laborious TCID50 assay. Also, analyses need to be performed regarding the quality of protein produced using this novel system to investigate proper post-translational modification. Regardless of these points of consideration, the data obtained thus far warrant further investigations into the robustness and safety aspects of this transient protein production system given that there is an increasing demand for high quality, high yield recombinant protein to facilitate pre-clinical and clinical pilot studies.

Essentially the process described in Figure 5 is not limited by scale given the calculations demonstrating that at 1,000 liter scale (PER.C6 cell density at 2 × 10^6 cells/mL = 2 × 10^{12} cells) a vector inoculation dose (50 IU/cell) of 1 × 10^{14} IU would be required. This amount of virus can easily be generated due to the efficient virus amplification using the suspension PER.E2A cell line cultured at 2 × 10^6 cells per mL in a 10 liter bioreactor within the timeframe of 4 weeks.

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