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The repression domain of the E1B 55 kDa protein participates in countering interferon-induced inhibition of adenovirus replication

Running Title: Ad5 E1B 55 kDa and repression of IFN-inducible genes. (52 characters)

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Abstract

To begin to investigate the mechanism by which the human adenovirus type 5 E1B 55 kDa protein protects against anti-viral effects of type 1 interferon IFN (Chahal, J.S., Qi, J. and S.J. Flint (2012) PLoS Pathogens 8 doi:10:1371), we examined the effects of precise amino acid substitution in this protein on resistance of viral replication to the cytokine. Only substitution by alanine of residues 443-448 (E1B Sub19) specifically impaired production of progeny virus, and resulted in a large defect in viral DNA synthesis in IFN-treated normal human fibroblasts. Untreated or IFN-treated cells infected by this mutant virus (AdEasyE1Sub19) contained much higher steady-state concentrations of IFN-inducible GBP1 and IFIT2 mRNAs than did wild type-infected cells, and of the corresponding newly transcribed pre-mRNAs, isolated exploiting 5′-ethynyluridine labeling and click chemistry. These results indicated that the Sub19 mutations impair repression of transcription of IFN-inducible genes by the E1B 55 kDa protein, consistent with their location in a segment required for repression of p53 dependent transcription. However, when synthesized alone, the E1B 55 kDa protein inhibited expression of the p53-regulated genes BAX and MDM2, but had no impact whatsoever on induction of IFIT2 and GBP1 expression by IFN. These observations correlate repression of transcription of IFN-inducible genes by the E1B 55 kDa protein with protection against inhibition of viral genome replication, and indicate that the E1B 55 kDa protein is not sufficient to establish such transcriptional repression.

(234 words)

Introduction

The E1B gene of species C human adenoviruses such as adenovirus type 5 (Ad5) encodes major, unrelated proteins of 19 and 55 kDa, each of which can cooperate with viral E1A gene products to transform rodent cells, and counter host cell responses detrimental to viral replication (4, 5). The E1B 19 kDa protein is a viral homolog of cellular anti-apoptotic proteins such as Bcl2, and blocks induction of apoptosis by the E1A proteins in transformed and infected cells (5, 14). The known protective functions of the E1B 55 kDa protein are fulfilled by a virus-specific E3 ubiquitin (Ub) ligase assembled from the E1B and the viral E4 Orf6 proteins, and the cellular proteins cullin5, elongins B and C, and Rbx1 (30, 65), which ubiquitinylates multiple cellular substrates to target them for subsequent proteasomal degradation. These substrates include the cellular tumor suppressor p53 (13, 30, 51, 65) and the Mre11, Rad50 and Nbs1 proteins that comprise the MRN complex (80). As the viral immediate early E1A 243R protein can induce apoptosis via stabilization of p53 (17, 49, 66, 86), removal of the latter protein as a result of the action of the E1B 55 kDa protein-containing E3 Ub ligase is thought to prevent induction of G1 arrest or apoptosis in infected cells (4, 5, 47). The proteins of the MRN complex recognize double-strand breaks in DNA to activate signaling pathways that result in repair by recombination or non-homologous end joining (NHEJ) (15, 45, 82, 90). It is well established that when MRN components are not targeted for degradation by the virus-specific E3 Ub ligase or relocalized by the viral E4 Orf3 protein (80, 81), viral DNA synthesis is impaired in infected cells (24, 43, 53). Furthermore, late in the infectious cycle concatamers of randomly orientated copies of the viral genome are formed in NHEJ-dependent reactions (23, 80, 91). Such concatamerization also requires the cellular enzyme DNA ligase IV (80), another substrate that is targeted for proteasomal degradation by the virus-specific E3 Ub ligase (3). Other cellular

proteins marked for degradation by this enzyme include Bloom helicase (61), and integrin α3, which may be removed from infected cells to facilitate release of progeny virus particles (16). The assembly of the E1B 55kDa and E4 Orf6 protein – containing E3 Ub ligase is also necessary for induction of selective export of viral late mRNAs from the nucleus (8, 93), one of the first functions in the infectious cycle to be ascribed to the E1B 55 kDa protein (64, 92).

In addition to its important functions as a component of the virus-specific Ub ligase, in which it is thought to serve as a substrate recognition subunit (7, 13, 51, 76), the E1B 55 kDa protein exhibits additional, E4 Orf6-independent activities. For example, it is also a Sumol E3 ligase (59, 63) that modifies p53 to induce association of this cellular protein with nuclear Pml bodies and its subsequent export from the nucleus (63). This mechanism of blocking regulation of transcription by p53 is thought to contribute to the ability of the E1B 55 kDa protein to cooperate with viral E1A proteins to transform rodent cells in culture (59, 63), as does a second E4 Orf6 protein-independent activity, inhibition of p53-dependent transcription. Early studies using transient expression assays established that the E1B 55 kDa protein is sufficient to repress expression of p53-dependent reporter genes (95). Mutations that result in impaired interaction of the E1B 55 kDa protein with p53 (39), impaired function of the repression domain (84, 97) or inhibition of sumoylation and nuclear entry of the E1B protein (22) inhibit E1B-55kDa protein-dependent transformation. Conversely, a greater degree of repression of p53-dependent transcription and more efficient transformation were observed when the intranuclear concentration of the E1B protein was increased by substitutions of critical residues within its nuclear export signal (21).

In principle, inhibition of p53-dependent transcription by the E1B 55 kDa protein could also represent a mechanism to ensure prevention of induction of G1 arrest or apoptosis during

the infectious cycle. However, this viral protein is not required for inhibition of expression of classic p53-dependent genes, such as p21 (CDKN1) and MDM2, in infected primary human epithelial cells or established cell lines (34, 60). Nor is apoptosis induced when the p53 protein accumulates to high concentrations in cells infected by E1B 55 kDa null mutants (9, 60). Furthermore, analysis of alterations in cellular gene expression by microarray hybridization demonstrated that infection of normal human cells with a mutant that cannot direct production of the E1B 55 kDa protein (Hr6) blocked the p53 transcriptional program as effectively as did Ad5 infection, even though over 600 cellular genes were observed to be differentially expressed in Hr6- and Ad5-infected cells (56). Subsequently, the E4 Orf3 protein has been reported to be responsible for blocking the transcriptional activity of p53 in infected cells (78).

The set of genes repressed by the E1B 55 kDa protein following infection of normal human cells is highly enriched for those associated with innate immune responses and anti-viral defense (56), notably 130 genes previously identified as inducible by interferon α or β (type I interferons, hereafter designated IFN (see Supplemtnal Table 1, 12). Indeed, replication of Hr6 in normal human fibroblasts or epithelial cells was observed to be several orders of magnitude more sensitive to exposure of cells to IFN than replication of Ad5 (12). The identical phenotype was exhibited by an E1B 55 kDa null mutant, AdEasyE1Δ2347 (11), engineered to contain the Hr6 mutation that prevents production of the E1B 55 kDa protein deletion of bp 2347 (41) but none of the other mutations recently identified in the Hr6 genome (40). Furthermore, the concentrations of pre-mRNAs synthesized from several IFN-inducible genes were increased substantially in cells infected by Hr6 and AdEasyE1Δ2347, compared to cells infected by the wild-type parental viruses, in the absence or presence of exogenous IFN (12). These observations indicate that the E1B 55 kDa protein makes an important contribution to the

resistance of adenoviral replication to IFN. The experiments reported here were undertaken to investigate further the mechanism by which this E1B protein blocks the action of IFN, in particular the relationship of this to the transcriptional repression function of the protein.

Materials and Methods

Cells and Viruses. Human 293 and A549 cells were maintained in monolayer culture in DMEM (Gibco-BRL) containing 5% calf serum (Gibco-BRL) and 5% Hyclone bovine growth serum (Thermo-Fisher Scientific). Normal human foreskin fibroblasts (HFFs) were maintained in the same medium supplemented with 7.5% Hyclone bovine growth serum.

Isolation of the E1B 55 kDa null mutant viruses AdEasyE1Δ2347 and AdEasyE1GΔ2347 has been described (11, 41). Mutations designed to result in precise substitutions of specific amino acids with gain or loss of a restriction endonuclease cleavage site were introduced by using the QuickChangeII site-directed mutagenesis kit (Stratagene-Agilent Technologies) into the coding sequence of the E1B 55 kDa protein present in the shuttle plasmids pShuttleE1 or pShuttle E1-G (41). The presence of an expression cassette for eGFP upstream of the viral E1A transcription unit in the latter plasmid (11) facilitated subsequent identification of mutant virus plaques. Following initial screening by cleavage of products of mutagenesis with the appropriate restriction endonuclease, the presence of the desired mutation(s) and absence of other changes in the E1B gene were confirmed by sequencing (Genewiz). The altered E1 regions were recovered into the viral genome by homologous recombination between the shuttle plasmids and pAdEasy (33) in *E. coli*, and mutant viruses isolated from these genomes, and validated as described previously (41, 56). The mutants and their wild-type parent, AdEasyE1 or AdEasyE1G, were propagated in 293 cells (28), and concentrations of infectious particles determined by plaque assay on these same cells.

Type I interferon (IFN) (PBL Interferon source) was diluted in phosphate-buffered saline containing 0.1% (w/v) bovine serum albumen (BSA)(Sigma-Aldrich). To assess sensitivity of

viral replication to IFN, HFFs were incubated with medium containing 250 units/ml IFN or only the BSA vehicle for 24 hrs. prior to infection with the multiplicities of wild-type or mutant viruses indicated. Following adsorption, incubation was continued in medium containing IFN or BSA only for various periods. Viral yields were determined by using plaque assays in 293 cells.

Isolation of HFFs stably expressing the Ad5 E1B 55 kDa protein coding sequence. The segment of the Ad5 genome from bp 2019 to 3601, which encompasses the complete E1B 55 kDa protein coding sequence, was amplified by PCR using FideliTaq (Affymetrix) according to the manufacturer's instructions and 0.2 μM of the primers forward: GCAGTTACCGGTGGAGCAGGAGCAATGGAGCGAAGAAAC and reverse: GCAGTCGAATTCTGCACCTGCACCTGCACCGCGGCTGCTGCTGCAAAA, which carry AgeI and EcoRI restriction endonuclease sites, respectively. This segment was ligated into the multiple cloning site of the pLJM1-eGFP plasmid (Addgene) (71) after removal of the eGFP coding sequence via AgeI and EcoRI digestion. The sequence of the E1B 55 kDa insert was verified by sequencing (Genewiz). The parental pLJM1-eGFP plasmid was used in parallel experiments as a control for exposure to lentivirus and transduction. Transducing lentivirus particles were generated by triple transfection of 293FT cells with either the pLJM1-eGFP control or pLJM1-E1B 55 kDa constructs with packaging (pCMV-dR8.91) and envelope (VSV-G) vectors (19). HFFs were transduced with these lentivirus preparations, and stable transductants selected by maintenance in medium containing 0.5 μg/ml puromycin (Sigma-Aldrich).

Immunoblotting. To examine accumulation of viral proteins, HFFs were infected with AdEasyE1, AdEasyE1G or the mutants indicated for increasing periods, and cell extracts prepared as described previously (27). Steady state concentrations of the viral E1A, E1B 55 kDa

proteins and late protein V were examined by immunoblotting with monoclonal antibodies M73 (31), 2A6 (73) and F58#1 (50), respectively, as described (27). Cellular β-actin was examined in parallel using a horseradish peroxidase-conjugated monoclonal antibody (Abcam) to provide an internal control. The cellular proteins p53 and Stat1 phosphorylated at Y701 were detected by using the mouse monoclonal antibodies DO1 conjugated to HRP (Santa Cruz) and 5806 (Cell Signalling, Inc.), respectively.

Immunofluoresence. The localization of the E1B 55 kDa protein in wildtype- and mutant-infected HFFs was examined using mouse monoclonal antibody 2A6 and AlexaFluor 488 anti-mouse IgG as described previously (27).

Measurement of viral DNA concentrations. HFFs at 80-90% confluence were infected with 3 pfu/cell AdEasyE1, AdEasyE1Δ2347 or AdEasyE1Sub19 for 2 or 72 hrs. Total cell DNA was isolated as described (11) and concentrations of viral DNA determined by using real time PCR and SBYRGreen (Applied Biosystems) detection of a 90 bpr amplicon in the major late transcription unit, with a cellular β-actin amplicon as internal control, exactly as described previously (12). All samples were assayed in triplicate.

Measurement of relative mRNA concentrations. HFFs were infected with 200 pfu/cell AdEasyE1, AdEasyE1Δ2347, or AdEasyE1-Sub19 for 48 hrs. Cells were harvested using TRIzol (Invitrogen), and whole-cell RNA prepared according to the manufacturer's instructions. Following resuspension in DNase I digestion buffer (Roche), the nucleic acid samples were incubated with 0.4 units/μl DNase I prior to phenol:$CHCl_3$ extraction and ethanol precipitation. RNA samples were resuspended in 10 mM Tris, pH 7.5, containing 5 mM NaCl, and 0.5 U/μl RNasin (Promega). RNA concentrations were determined from A_{260} readings made using a

NanoDrop ND-1000 spectrophotometer. cDNA was synthesized from RNA by priming with 200 ng random hexamers (Roche) per 1 µg of RNA, and extension with SuperScript II reverse transcriptase (Invitrogen) using the conditions recommended by the manufacturer. Quantitative real-time PCR was carried out using the ABI PRISM 7900HT sequence detection system with SYBR Green Master Mix and SDS version 2.1 software (Applied Biosystems). For IFIT2 mRNA, the PCR primers were; fwd: AATTGAGGTGGCAACATAGTTTGA rev: CCCGTCGCTTCTAGCTATGTATC; for GBPl mRNA, fwd: GTCAACGGGCCTCGTCTAGA, rev: CCCACTGCTGATGGCAATG; for BAX mRNA, fwd: TTTCTGACGGCAACTTCAACTG rev: GGTGCACAGGGCCTTGAG; for hMDM2 mRNA, fwd: TCCTCTCAAGCTCCGTGTTTG; rev: TCATGATGTGGTCAGGGTAGATG; and for the β-actin internal control fwd: TCCTCCTGAGCGCAAGTACTC, rev: ACTCGTCATACTCCTGCTT. Relative amplicon concentrations were determined by the standard curve method using a 10-fold dilution series of the IFN-treated, AdEasyE1Δ2347-infected HFF cDNA as the standards for GBP1 and IFIT2, and DNA of a recombinant human cytomegalovirus BAC containing the genomic human β-actin sequence (a kind gift of Thomas Shenk) as the standard for β-actin.

Isolation of newly synthesized RNA. HFFs at 80-90% confluence were infected with 50 pfu/cell AdEasyE1 or AdEasyE1Sub19, or mock infected. The medium was replaced with DMEM containing 0.5 mM 5-ethynyluridine (5-EU) (Invitrogen), and incubation at 37°C continued for an additional 40 minutes. The cells were then harvested and total RNA purified as described in the previous paragraph. The RNA newly labeled with 5-EU was biotinylated using click chemistry and isolated by binding to streptavidin attached to magnetic beads using a click-iT nascent RNA capture kit (Invitrogen) following the protocol recommended by the manufacturer.

Complementary DNA was synthesized from the newly synthesized RNA populations isolated from equal numbers of cells infected by the different viruses as described above, and IFIT2 and IL6 pre-mRNAs detected by PCR with the primers spanning exon-intron junctions described previously (11). Viral E1A pre-mRNA was examined using such pre-mRNA-specific PCR primers with sequences fwd; GACCTGTGGCATGTTTGTCTACA and rev CACCAAACCCACCACTCTATCA while β-actin mRNA was detected with the primers described in the previous section. All PCR products were examined by electrophoresis in 8% polyacrylamide gels cast and run in 40 mM Tris-acetate, pH 8.3, containing 1 mM EDTA followed by staining with 2.5 μg/ml ethidium bromide.

Results

Identification of substitutions within the E1B 55 kDa protein that render viral replication sensitive to IFN.

Previous studies have suggested that the Ad5 E1B 55 kDa protein does not comprise discrete structural and functional domains, as the stability, localization and interactions of the protein are sensitive to even relatively small insertions at multiple sites throughout the coding sequence (6, 27, 96). To identify sequences required for this protein to confer resistance of viral replication to IFN (12), we therefore introduced mutations that result in precise amino acid substitutions. These mutations were designed to alter previously described sequence motifs, residues reported to be required for specific functions of the protein, and/or sequences predicted to be exposed on the surface of the protein (Table 1). Mutations were introduced into the E1B 55 kDa protein coding sequence in shuttle plasmids that contain both the E1A and E1B genes

(11, 41) and recovered into viral genomes prior to isolation, validation and propagation of mutant viruses as described in Materials and Methods. Four of the mutations were introduced into a viral genome that contains an expression cassette for GFP upstream of the E1A transcription unit (11). To assess the effects of the mutations on sensitivity of viral replication to IFN, HFFs were incubated with 250 units/ml IFN or BSA-only control for 24 hrs. prior to and following infection. Cells were infected in parallel with 3 pfu/cell of the substitution mutants and the corresponding phenotypically wild-type parent and E1B 55 kDa-null mutant viruses: as summarized in Table 1. Viral yields were determined 72 hrs. after infection, as described in Materials and Methods. The results of the initial screen based on two independent infections with each mutant are summarized in Table 1.

It is well established that replication of wild-type Ad5 is quite refractory to inhibition by IFN (2, 12, 89). The majority of the E1B 55 kDa substitution mutations did not reduce such resistance significantly: the ratios of viral yields in the presence compared to in the absence of IFN were within a factor of two of the values observed for the corresponding wild-type virus (Table 1). However, the replication of three mutants in the presence of IFN was observed to be decreased to a greater degree than that of the wild-type (Table 1). Replication of AdEasyE1GSub7 and AdEasyE1Sub17 was 25-fold more sensitive to IFN than that of their wild-type parents, whereas AdEasyE1Sub19 replication was some 10-fold more sensitive. To determine whether any of these defects were a trivial consequence of alterations in protein concentration, the steady-state concentrations of the viral E1B 55 kDa proteins were compared 24 hrs. after infection with 50 pfu/cell mutant or wild-type virus by immunoblotting. Viral E1A proteins were examined in parallel, to provide an internal control. As summarized in Table 1, the substitutions introduced into AdEasyE1Sub7 reduced substantially the steady-state

concentration of the E1B 55 kDa protein in infected cells, with no effect on E1A protein accumulation (data not shown). Two of the three substitutions present in this mutant replace large hydrophobic residues with Ala (Table 1), consistent with the conclusion that the stability of this altered E1B protein is reduced. Regardless, the very low quantities of the E1B 55 kDa protein produced in AdEasyE1Sub7-infected cells account for the sensitivity of the replication of this mutant to IFN. The accumulation of the E1B 55 kDa protein was also observed to be reduced in cells infected for 24 hrs. with AdEasyE1GSub17 compared to its wild-type parent, but by 36 hours after infection, the concentration of this altered E1B protein increased significantly (Table 1; data not shown). These observations suggested that the increased sensitivity to IFN of replication of this mutant observed in the low multiplicity of infection screen (Table 1) was at least in part the result of delayed accumulation of the E1B 55 kDa protein. Consistent with this view, replication of this mutant was considerably less sensitive to IFN when HFFs were infected at high multiplicity (50 pfu/cell) (data not shown).

As summarized in Table 1, replication of AdEasyE1Sub19 exhibited an increase in sensitivity compared to the wild-type parent of some 10-fold, without a defect in the accumulation of the E1B 55 kDa protein. Indeed, increased steady-state concentration of both this and the E1A proteins were observed in mutant virus-infected cell (see e.g. Fig 2A). As these initial observations indicated that the enhanced sensitivity of replication of this mutant to IFN is not a trivial consequence of failure to synthesize stable E1B protein, we examined the properties of this mutant in more detail. We first compared its sensitivity to IFN to that of the corresponding E1B 55 kDa null mutant, AdEasyE1Δ2347 (41), following infection of HFFs at high multiplicity (50 pfu/cell) for 36 hrs., or at low multiplicity (3 pfu/cell) for 88 hrs. The former condition corresponds to a single infectious cycle with the great majority of cells initially

infected (26). In contrast, the low multiplicity protocol allowed for spread of infection from the small number of cells infected initially, and is therefore likely to be more representative of infection and the impact of host anti-viral defenses *in vivo*. In agreement with our previous studies (12), replication of AdEasyE1Δ2347 was much more sensitive to inhibition by IFN than that of its phenotypically wild-type parent, particularly following low multiplicity infection (Fig. 1). The 2 to 3 orders of magnitude decreases in yield of this null mutant observed when cells were not exposed to IFN are also consistent with the requirement for the E1B 55 Da protein to allow maximally efficient viral DNA synthesis in HFFs (11, 26). In the absence of IFN, no significant impact of the Sub19 E1B substitutions on virus yield was observed (Fig. 1). However, replication of this mutant was more sensitive to IFN-induced inhibition than that of the wild-type following infection at either high or low multiplicity, although less sensitive than replication of AdEasyE1Δ2347 (Fig. 1). For example, following low multiplicity infection the ratio of yields of AdEasyE1Sub19 in the presence and absence of IFN was some 13-fold lower than that of the wild type. We therefore next assessed the effects of the E1B Sub19 substitutions on expression of viral genes and genome replication.

The Sub19 substitutions impair viral genome replication only in IFN-treated HFFs.

To examine expression of viral early and late genes, HFFs were infected with 30 pfu/cell AdEasyE1, AdEasyE1Δ2347 or AdEasyE1Sub19 for increasing periods, or mock-infected, and the accumulation of immediate early E1A proteins, the early E1B 55 kDa protein and late protein V monitored by using immunoblotting as described in Materials and Methods. As expected (41), the E1B 55 kDa protein could not be detected at any time after infection with AdEasyE1Δ2347,

293 and production of late protein V was severely impaired (Fig. 2A). Higher concentrations of the
294 E1A and E1B 55 kDa proteins were observed at 20 and 40 hrs. after infection with
295 AdEasySub19 than in cells infected in parallel by the wild-type, and of late protein V by 40 hrs.
296 p.i. (Fig. 2A). Quantification of the E1B 55 kDa protein signal at 20 hrs. p.i., using β-actin as the
297 internal control, indicated that the E1B protein accumulated to a 10-fold higher concentration in
298 AdEasyE1Sub19- compared to wild-type- infected cells. This value was in excellent agreement
299 with the 11-fold increase measured in an independent experiment, in which dilutions of extracts
300 of AdEasyE1Sub19-infected cells harvested 24 hrs. after infection were compared to the
301 AdEasyE1-infected cell extract, again using β-actin as an internal control (data not shown). We
302 attribute the increased concentrations of all viral proteins examined in AdEasyE1Sub19-
303 compared to wild type- infected cells (Fig. 2A) to underestimation of the titer of the mutant
304 virus, which formed exceptionally small plaques on complementing 293 cells. Regardless, it is
305 clear that the increased sensitivity of replication of AdEasyE1Sub19 to IFN-induced inhibition is
306 not merely an uninteresting consequence of destabilization of the E1B 55 kDa protein.
307 Furthermore, like the wild type, the altered E1B 55 kDa protein synthesized in AdEasyE1Sub9-
308 infected cells was concentrated in nuclei (Fig. 2B).

309 We have reported previously that when the E1B 55 kDa protein is not present during
310 infection of normal human cells, exposure to exogenous IFN induces inhibition of viral DNA
311 synthesis (12). The effect of the Sub19 substitutions on this reaction in the infectious cycle was,
312 therefore, examined by using quantitative PCR as described in Materials and Methods.
313 Consistent with our previous observations (12), replication of the E1B 55 kDa null mutant
314 AdEasyE1Δ2347 genome was impaired in HFFs that were not treated with IFN (Fig. 2C).
315 However, replication of this mutant genome was inhibited far more severely, compared to that of

316 the wild type, when HFFs were exposed to IFN (Fig. 2C), as was previously observed in normal
317 human bronchial/tracheal epithelial cells (NHBECs) (12). In the latter experiments, the majority
318 of cells were infected using a relatively high multiplicity for a period (24 hrs) insufficient to
319 allow significant release of progeny virus particles. In contrast, HFFs, which are poorly infected
320 by Ad5 (26), were infected with a multiplicity (3 pfu/cell) at which only some 5% of cells
321 become infected for a period longer than the infectious cycle. This protocol, which permits
322 secondary infection and hence amplification of any inhibitory effects of IFN, seems likely to
323 account for the greater degree of IFN-induced inhibition of viral DNA synthesis observed in both
324 E1B 55 kDa null mutant and wild type-infected HFFs (Fig 2C) than previously in NHBECs (11).

325 No significant difference in the efficiency of viral DNA synthesis was detected in
326 AdEasyE1Sub19- compared to AdEasyE1- infected cells that were not treated with IFN (Fig.
327 2C). However, replication of this mutant genome was considerably more sensitive to inhibition
328 in IFN treated cells, exhibiting a decrease some 300-fold greater than that of the wild type (Fig.
329 2C). In these experiments, increases in viral DNA concentration in infected cells were calculated
330 relative to the input value measured 2 hrs after infection, an approach that accounts for any
331 differences in the quantities of viral DNA entering wild type- and mutant- infected cells. This
332 value was, in fact, some 5-fold higher in cells infected by AdEasyE1Sub19 compared to those
333 infected by AdEasyE1, consistent with the results of analysis of viral early proteins described
334 previously. Replication of this mutant was also observed to some 300-fold more sensitive to
335 exogenous IFN than that of AdEasyE1 when cells were infected with equal numbers genomes,
336 and mutant-infected cells did not accumulate higher concentrations of viral E1A proteins than
337 wild type-infected cells (data not shown). We therefore conclude that replacement residues

R443-R448 by Ala in the Sub19 E1B 55 kDa protein results in impaired viral genome replication specifically in cells exposed to IFN.

The Sub19 substitutions impair repression of IFN-inducible genes by the E1B 55 kDa protein.

When fused to the DNA-binding domain of the *S. cerevisiae* transcriptional activator Gal4, the E1B 55 kDa protein can act as a strong repressor of transcription from promoters that contain Gal4 binding sites (97). Such repression is severely impaired by the insertion of 4 amino acids at R443 in the E1B protein (97), the first of the 6 charged or hydrophilic residues replaced by Ala by the Sub19 mutations (Table 1). Our previous studies have established that the E1B 55 kDa protein inhibits expression of over 100 IFN-inducible genes, and synthesis of pre-mRNA from such genes (12, 56). As it was therefore of considerable interest to investigate whether the Sub19 substitutions had any impact on this function of the E1B protein, we compared expression of IFN-inducible genes in cells infected by the wild type and mutant viruses.

HFFs exposed to IFN or vehicle only control were infected with AdEasyE1, AdeasyE1Δ2347 or AdEasyE1Sub19 for 30 hrs., or mock-infected. To allow a quantitative comparison, cDNAs were synthesized from total cell RNA using random priming, and their relative concentrations measured by quantitative PCR as described in Materials and Methods, with primer pairs specific for mRNAs encoded by the IFN-inducible GBP1 and IFIT2 genes, or, as an internal control, for β-actin mRNA. Typical results of these experiments are shown in Figure 3. Exposure of mock-infected cells to IFN induced substantial increases in the concentrations of both GBP1 and IFIT2 mRNAs, of some 30- and 100- fold respectively (Fig. 3). The low levels of IFIT2 mRNA observed in mock-infected cells in the absence of IFN treatment

(Fig. 3B), and the greater induction of synthesis of this than of GBP1 mRNA by IFN (Figs. 3A and B) are in excellent agreement with our previous qualitative analyses of the responses of HFFs and NHBECs to IFN (12). The expression of the IFN-inducible genes was not decreased in wild type- compared to mock- infected cells (Fig. 3), as we observed previously in normal human cells both untreated and exposed to IFN (12). This difference can be attributed to infection until significantly later in the late phase of infection (47 hrs. p.i.) in these compared to previous (24 hrs. p.i.) experiments: at such very late times of infection, expression of IFN-inducible genes increases significantly in wild type- infected cells (D. Miller; B. Rickards and S.J.F. unpublished observations). Nevertheless, consistent with our previous observations summarized above, both these IFN-inducible mRNAs accumulated to higher concentrations in both untreated and IFN treated cells infected with the E1B 55 kDa null mutant than in cells infected by its wild-type parent (Fig. 3). The same pattern was observed in AdEasyE1Sub19-infected cells exposed to IFN (Fig. 3), indicating that substitution of residues 443-448 impairs the ability of the E1B 55 kDa protein to block expression of genes in response to IFN. However, synthesis of the Sub19 E1B 55 kDa protein in cells that were not treated with IFN led to accumulation of significantly higher concentrations of GBP1 and IFIT2 mRNAs than observed in null mutant-infected cells (Fig. 3).

This disparity between the phenotypes exhibited the null and substitution mutants might appear paradoxical. However it can be explained by differences in another important function of the E1B 55 kDa protein during the adenoviral infectious cycle, induction of selective export of viral late mRNAs from the nucleus during the late phase of infection, and hence maximally efficient expression of viral late genes (6, 64, 92): cells infected by the AdEasyE1Δ2347 mutant lack the E1B 55 kDa protein and hence selective regulation of mRNA export, whereas the E1B

Sub19 substitutions do not impair this function, as indicated by the absence of defects in viral late gene expression (Fig. 2C). Furthermore, the virus-specific E3 ubiquitin ligase, which is required for selective export of viral late mRNAs (8, 93), assembles efficiently in AdEasyE1Sub19- infected cells (Fig. 5). The set of mRNAs selectively exported in an E1B 55 kDa protein-dependent manner during the late phase of infection includes not only viral late mRNAs, but also mRNAs encoded by cellular genes transcriptionally activated during the late phase of infection (25, 57, 94). In HFFs not exposed to IFN, this latter class includes the IFN-inducible genes repressed by the E1B 55 kDa protein (56; J.S.C. and S.J.F., unpublished observations). As such mRNAs that are not exported selectively do not accumulate in the nucleus (94), their concentration would be expected be lower when the E1B 55 kDa protein does not induce selective export than when it does, as indeed observed for the representative IFN-inducible mRNAs in AdEasyE1Δ2347- compared to AdEasyE1Sub19- infected cells (Fig. 3).

We have reported previously that the concentrations of the pre-mRNAs synthesized from several IFN-inducible genes are increased in infected HFFs (and NHBECs) when the E1B 55 kDa protein is not present (12). This observation, in conjunction with the significant stimulation of expression of such genes induced by the E1B Sub19 mutations that alter the previously described transcriptional repression domain, suggested that the E1B 55 kDa protein acts as a repressor of transcription of IFN-inducible genes. To test this inference directly, we exploited pulse-labeling of RNA with 5-ethynyluridine and subsequent click chemistry (36, 83) for attachment of biotin to isolate newly-transcribed RNA as described in Materials and Methods. HFFs infected with 50 p.f.u./cell AdEasyE1 or AdEasyE1Sub19 for 24 hrs, or mock-infected, were exposed to the uridine analogue for 40 minutes, a period sufficient to complete synthesis and processing of pre-mRNAs, but short enough to minimize the impact of any differences in

406 turnover. The concentrations of pre-mRNAs, as well as of control β-actin mRNA, were
407 compared by PCR, following reverse transcription using random priming of newly-synthesized
408 RNA isolated from the same number of cells. The concentrations of β-actin mRNA in these
409 populations were similar in mock, wild-type- and mutant- infected cells (Fig 4). Consistent with
410 levels of viral proteins described previously (Fig 2A), somewhat higher concentrations of E1A
411 pre-mRNA were observed in cells infected by the mutant virus (Fig. 4). Consistent with
412 previous observations (12), significantly more IFIT2 and IL6 pre-mRNAs was detected in
413 AdEasyE1Sub19- compared to AdEasyE1- infected cells (Fig 4).

414 **The E1B 55 kDa protein does not prevent activation of Stat1.**

415 Binding of exogenous IFN to its heterodimeric receptor initiates a signal transduction
416 cascade to result in phosphorylation of Stat1 and Stat2 at specific tyrosine residues, and their
417 entry into the nucleus and formation of the sequence-specific activator of transcription of IFN-
418 inducible genes Isgf3 by association with Irf9 (67, 70, 74). As the E1B 55 kDa protein acts
419 upstream of transcription of such genes, we assessed the impact of this protein on activation of
420 Stat1. HFFs were infected with AdEasyE1, AdEasyE1Δ2347 or AdEasyE1Sub19 for increasing
421 periods, and the accumulation of Stat1 phosphorylated on Tyr701 examined by immunoblotting.
422 Extracts of mock-infected cells treated with 250 units/ml IFN for 40 hrs, or untreated, were
423 examined in parallel to provide a positive control for induction of Stat1 phosphorylation. As
424 described previously, somewhat higher concentrations of the E1A and E1B 55 kDa proteins were
425 observed in cells infected by AdEasySub19, compared to its wild type parent (Fig. 5). In wild-
426 type- infected cells, Tyr701-phosphorylated Stat1 attained a level only slightly lower than that
427 observed in IFN-treated, uninfected cells, but not until between 20 and 40 hrs after infection
428 (Fig. 5). Neither the absence of the E1B 55 kDa protein nor the presence of higher than wild-

type concentrations of the Sub19 derivative that cannot block expression of IFN-inducible genes (Figs 3 and 4) induced reduced phosphorylation of Stat1 (Fig. 5). In fact, this modification was accelerated in AdEasyE1Sub19-infected cells, in parallel with the alterations in expression of viral immediate early and early genes (Fig 5). Nor could any differences in the largely nuclear localization of Stat1 phosphorylated on Tyr701 be discerned in cells infected by AdEasyE1Sub19- and AdEasyE1- infected cells (data not shown).

We also wished to investigate the impact, if any, of the E1BSub19 alterations on the assembly of the Ad E3 Ub ligase, and therefore also examined the concentrations of a classic substrate of this enzyme, p53 (see Introduction) by immunoblotting. As expected, p53 accumulated to high concentrations in cells infected by the E1B 55 kDa null mutant, but was barely detectable in cells infected with either the wild type virus or AdEasyE1Sub19 (Fig 5). As different sequences of the E1B 55 kDa protein mediate recognition of different substrates of the Ad E3 Ub ligase (41, 76), it remains possible that substitution of residues 443 to 448 by Ala impairs effects on one or more other substrates. Nevertheless, this observation indicates that the E1BSub19 mutations do not impair assembly or catalytic activity of this enzyme.

The E1B 55 kDa protein is not sufficient to repress expression of IFN-inducible genes.

We have reported previously that mutations that prevent assembly of the virus-specific E3 ubiquitin ligase that contains the E1B 55 kDa and E4 Orf6 proteins do not increase the sensitivity of viral replication to inhibition by exogenous IFN (12). These observations establish that the ability of the E1B 55 kDa protein to block inhibitory effects of IFN, in contrast to the majority of its functions during the infectious cycle (see Introduction,) does not depend on interaction with the E4 Orf6 protein, but provide no information about whether other viral

proteins might be required. As the E1B 55 kDa protein alone can repress transcription of appropriate reporter genes in *in vitro* and transient expression assays (52, 84), it was of particular interest to determine whether it were also sufficient to inhibit expression of IFN-inducible genes in normal human cells.

To address this question, derivatives of HFFs that stably express the E1B 55 kDa protein, or as a control, eGFP, coding sequence under the control of the human cytomegalovirus immediate early enhancer/promoter were isolated by puromycin selection following transduction with lentirviral vectors, as described in Materials and Methods. Expression of the exogenous genes was then examined by immunoblotting of total cell extracts. The E1B 55 kDa protein was readily detected in HFFs transduced with vector carrying the expression cassette for this protein (HFF-E1B cells), as was eGFP in the control, HFF-GFP cells (Fig. 6A). To determine whether the E1B protein made in HFFs in the absence of other viral proteins were functional, its ability to complement the defect in genome replication in IFN-treated cells infected by the E1B 55 kDa null mutant was assessed. HFF-E1B or control HFF-GFP cells were exposed to exogenous IFN or BSA only control, as described previously, and infected with 3 pfu/cell AdEasyE1 or AdEasyE1Δ2347, and the accumulation of viral DNA at 40 hrs. after infection measured by quantitative PCR. In HFF-GFP cells infected by the null mutant, synthesis of viral DNA was reduced somewhat in the absence of IFN treatment, and very severely when the cells were exposed to this cytokine (Fig. 6B) exactly as observed in the parental HFFs (Fig. 2B). In contrast, no significant differences in the efficiencies of replication of mutant and wild-type genomes were observed in infected HFF-E1B cells (Fig. 6B). Such efficient complementation of the defects in viral genome replication characteristic of AdEasyE1Δ2347-infected, IFN-treated

HFFs established unequivocally that the E1B 55 kDa protein made in HFF-E1B cells is functional, and present at sufficient concentrations.

We therefore next compared the effect of exogenous IFN on expression of IFN-inducible genes in HFF-E1B and HFF-GFP cells. Cells were incubated with 250 units/ml IFN, or BSA only control, for 8.5 hrs. and the concentrations of GBP1 and IFIT2 mRNAs, relative to that of β-actin mRNA, measured as described in a previous section. In HFF-GFP cells, IFN induced large increases in expression of both the GBP1 and IFIT2 genes, and closely similar increases were observed in HFF-E1B cells (Figs 6C and D). For example, the concentrations of GBP1 mRNA were increased 11.6- and 14.5-fold following IFN treatment of HFF-GFP and HFF-E1B cells, respectively. We therefore conclude that, when synthesized in the absence of other viral proteins, the E1B 55 kDa protein cannot repress expression of IFN inducible genes.

In parallel, we compared expression of the p53-regulated BAX and MDM2 genes in HFFs that produce the E1B 55 kDa protein or eGFP. Even though untreated, uninfected HFFs contain low concentrations of p53 (9), the concentration of BAX and MDM2 mRNAs were some 2-fold lower in HFF-E1B than in HFF-GFP cells (Fig. 6E).

Efficient viral DNA synthesis in A549 cells exposed to interferon does not require the E1B 55 kDa protein.

The phenotypes exhibited by AdEasyE1Sub19 correlate the ability of the E1B 55 kDa protein to protect against IFN-induced inhibition of viral genome replication with repression of expression of IFN-inducible genes. The set of 300 genes repressed by the E1B 55 kDa protein in HFFs (56) contains 130 identified as IFN-inducible, including several associated with induction of apoptosis (12). However, as IFN-induced inhibition of viral replication in the absence of the

E1B protein cannot be attributed to programmed cell death (12), the relevant gene(s) remain unknown. To facilitate identification of these genes or their products, we wished to identify lines of established human cells in which replication of the E1B 55 kDa null mutant virus is inhibited by exogenous IFN. We therefore examined the effects of IFN treatment on replication of AdEasyE1, AdEasyE1Δ2347 and AdEasyE1Sub19 genomes in human cell lines commonly used in studies of adenovirus replication and known to be sensitive to IFN, namely HeLa (44) and A549 (2) cells. In the former, replication of the mutant virus genomes was 70 fold lower than that of the wild type following infection of cells exposed to IFN at a multiplicity of 1 p.f.u./cell (data not shown).

Treatment of A549 cells with 1000 units/ml IFN using the protocol described above for HFFs had no significant effect on accumulation of wild type genomes (Fig. 7). Remarkably, IFN treatment induced only 5- and 2-fold reductions in the quantities of viral DNA molecules synthesized in AdEasyE1Δ2347- and AdEasyE1Sub19, -infected A549 cells, respectively (Fig. 7). As these differences are insubstantial in comparison to those of several orders of magnitude resulting from these E1B mutations in HFFs (Fig. 2B), we conclude that the E1B 55 kDa protein is not necessary to prevent IFN-induced inhibition of viral genome replication in A549 cells. These cells are not simply refractory to typeI IFN, as exposure to this cytokine activates expression of numerous IFN-inducible genes (72) . It seems likely that these tumor- derived cells physically or functionally lack one or more IFN-inducible gene products that inhibit viral genome replication, and that are targeted by the E1B 55 kDa protein.

Discussion

Although no structural information about the multi-functional species C adenovirus E1B 55 kDa protein is available, a considerable body of information about sequence motifs, sites of post-translational modification and sequences required for interaction with various viral or cellular protein has been collected (reviewed in (6)). None of the substitution mutations in the E1B protein coding sequence that altered such previously characterized features, including the nuclear export signal, RNP motif and C-terminal sites of phosphorylation, examined in these experiments specifically impaired viral replication in normal human cells exposed to IFN (Table 1). In conjunction with our previous observation that mutations that prevent assembly of the virus-specific E3 ubiquitin ligase in infected cells also fail to render viral replication sensitive to exogenous IFN (12), these data suggest that the E1B 55 kDa protein blocks the anti-viral action of IFN by a mechanism that does not depend on previously described properties of the protein. Several Ala substitutions with segments of the E1B 55 kDa protein predicted to be surface exposed and not previously studied, such as the alterations in the N-terminal segment that also encodes the C-terminus of the E1B 19 kDa protein in a different reading frame introduced by the Sub1, 2, and 3 mutations, were also without effect (Table 1). In fact, only the replacement with Ala of residues 443 to 448, a run of 6 charged or hydrophilic residues, by the E1B Sub 19 mutations increased the sensitivity of viral replication to IFN (Fig. 1) independently of any reductions in accumulation of the altered protein (Fig. 2A). As mentioned previously, estimation of the impact of the Sub19 mutations in viral yield in IFN-treated cells was complicated by the very small plaque phenotype exhibited by AdEasyE1Sub19 (see Results), which suggests that this altered E1B 55 kDa protein acts as a dominant negative. This interpretation is consistent with both the larger quantities of the Sub19 E1B protein than of the endogenous wild-type protein likely produced in AdEasyE1Sub19- infected 293 cells (Fig. 2A) and the ability of the

E1B 55 kDa protein to self-associate when synthesized in the absence of other viral proteins (52, 58). Regardless, measurement in the increase in concentration of viral genomes over the value determined soon after entry (2 hr. p.i.), a parameter that is independent of any differences in the numbers of infecting particles or genomes, established that the E1B Sub19 mutations result in a severe defect in viral DNA synthesis specifically in IFN-treated cells (Fig. 2C).

We have reported previously that expression of some 130 IFN-inducible genes is increased significantly when the E1B 55 kDa protein is not present in infected cells, as are the concentrations of several pre-mRNAs synthesized from such genes (12, 56). The large increases in accumulation of IFN-inducible mRNAs in untreated or IFN-treated cells infected with AdEasyE1Sub19 over those measured in AdEasyE1-infected cells (Fig. 3) suggested that protection of viral replication and DNA synthesis against IFN-induced inhibition depends on transcriptional repression by the E1B protein: insertion of 4 amino acids at R443, the first residue replaced by Ala in the Sub19 E1B protein (Table 1), impairs both repression of p53-dependent transcription and the ability of an E1B 55 kDa-Gal4 DNA-binding domain fusion protein to act as a direct repressor of transcription in transient expression assays (95, 97). The increases in concentrations of newly-synthesized, IFN-inducible pre-mRNAs observed in AdEasyE1Sub19- compared to AdEasyE1-infected cells (Fig 4) provides direct experimental support for the conclusion that the E1B 55 kDa protein represses transcription of specific genes in infected normal human cells.

Although the mechanism of such repression is not yet known (see below), it can be distinguished in several respects from that proposed for inhibition of transcription of p53-regulated genes (5, 97), which has been implicated in the ability of the E1B 55 kDa protein to cooperate with viral E1A proteins in transformation of rodent cells (see Introduction).

Substitutions of the C-terminal sites of phosphorylation of the Ad5 or Ad12 E1B protein reduced its ability to inhibit p53-dependent transcription, or to act as a repressor when fused to an heterologous DNA-binding domain (84, 85, 99). However, such mutations induced neither increased expression of E1B 55 kDa protein-repressed genes in infected HFFs (56) nor increased sensitivity of viral replication (Table 1) or DNA synthesis (data not shown) to IFN. The E1B protein alone is sufficient to repress p53-depdent transcription in cells transiently or stably synthesizing the viral protein (32, 79, 84, 85, 95, 97, 99). The E1B 55 kDa protein fully competent to rescue the defects in genome replication of the null mutant AdEasyE1Δ2347 when stably produced in HFFs (Fig. 6B) also inhibited accumulation of mRNAs encoded by the p53-regulated BAX and MDM2 genes (Fig 6E). In contrast, it had no effect whatsoever on activation of expression of IFN-inducible genes by exogenous IFN (Figs 6C and D). It therefore appears that the E1B 55 kDa protein can inhibit transcription of cellular genes by multiple mechanisms, depending on whether it is synthesized in the absence of other viral proteins or in the context of infected cells.

The impaired repression of transcription of IFN-inducible genes in cells infected by AdEasyE1Δ2347 or AdEasyE1Sub19 could not be attributed to alterations in the kinetics or degree of activation of Stat1 (Fig 5), or its nuclear localization (data not shown), suggesting that the E1B 55 kDa protein does not target the signal transduction pathway responsible for activation of transcription of IFN-inducible genes. However, it is not clear whether this protein acts directly to repress transcription of IFN-inducible genes, as it can in *in vitro* or transient expression assays. The proximity of the E1B Sub19 substitutions to the R443 insertion in the previously described repression domain is consistent with such a mechanism, and the well characterized E1B-protein-containing E3 ubiquitin ligase is dispensable for protection against

IFN-induced inhibition of viral replication (11). On the other hand, mutations that prevent binding by the only motif in the protein implicated in interaction with nucleic acids, the RNP motif (35), did not reduce the resistance of viral replication to IFN (Table 1). Nor did analysis of the clusters of genes differentially expressed in cells infected by Ad5 and an E1B 55 kDa-null mutant (56) by using FIRE (20) identify any sequence motif(s) common to or overrepresented among, the promoters of E1B-repressed genes (data not shown). Furthermore, the E1B 55 kDa protein has been demonstrated more recently to function as Sumol E3 ligase (59, 63), and could therefore regulate transcription indirectly via this activity.

The failure of the E1B protein to block activation of expression of IFN-inducible genes in response to exogenous IFN when synthesized in uninfected HFFs (Fig. 6) implies that either one or more additional viral gene products or, perhaps less likely, alterations in the host cell environment induced by infection are also required. Analysis of protein associated with the E1B 55 kDa protein by mass spectrometry identified the viral IVa_2 and L4 100 kDa proteins (30). The E1B –IVa_2 protein interaction was confirmed by co-immunoprecipitation (30), but seems unlikely to contribute to inhibition of expression of IFN-inducible genes: although this protein possesses sequence-specific DNA binding activity, it contributes to activation of transcription from the viral major late promoter (62, 87). Furthermore, only 15 cellular genes were found to carry sequences corresponding to that recognized by the IVa_2 protein between positions -1000 and +500, and none were repressed by the E1B 55 kDa protein (55, 56). The small RNA VA-RNAI was the first of several adenovirus-encoded inhibitors of the anti-viral effects of IFN to be identified (42). However, this RNA acts downstream of expression of IFN-inducible genes by blocking activation of a specific effector of the IFN response, interferon induced double-stranded RNA activated protein kinase E1 (E1F2AK2, aka Pkr) (54). The viral E1A proteins prevent

609 inhibition of replication of vesicular stomatitis virus by exogenous IFN (2) and inhibit the
610 activation of transcription of IFN-inducible genes (1, 29, 68). Such inhibition is the result of
611 suppression of the Jak-Stat signaling pathway that leads to assembly in the nucleus of the crucial
612 activator Isgf3 (37, 38, 48, 77), and also sequestration of the coactivator p300 (69). Although the
613 E1A proteins are potent repressors of expression of IFN-inducible genes when synthesized in the
614 absence of any other viral proteins (1, 29, 38, 68), they are incapable of maintaining suppression
615 of expression of such cellular genes (Fig. 3), even when produced in large quantities in
616 AdEasyE1Sub19- infected cells (Figs. 2A and 5).

617 Like the E1B 55 kDa protein (Fig. 2; 12), the viral E4 Orf3 protein prevents inhibition of
618 viral genome replication in cells exposed to exogenous IFN (89). This protective function of the
619 E4 Orf3 protein, which has long been known to reorganize components of Pml nuclear bodies,
620 including the Pml protein, into track-like structures (10, 18, 46), becomes dispensable when
621 production of Pml or Daxx is impaired by RNA interference (88). Although the relocalization of
622 Pml and association with the E4 Orf3 protein are indistinguishable in IFN-treated (or untreated)
623 HFFs infected by wild type and E1B 55 kDa null mutant viruses (12), it is possible that the E1B
624 protein functions downstream of E4 Orf3- dependent Pml body disruption. Indeed, IFN-treated
625 cells infected by E1B 55 kDa null or E4 Orf3 null mutants display a strikingly similar failure in
626 the formation of viral replication centers (12, 89). Furthermore, the E1B protein has been
627 reported to interact with both E4 Orf3 (46) and Daxx (98), and to induce proteasomal
628 degradation of the latter by a mechanism that does not require the Ad E3 ubiquitin ligase (75).
629 The species C human adenovirus E4 orf3 protein also sequesters components of the Mre11-
630 Rad50-NbS1 (MRN) double strand break repair complex into the track like structures (23, 80,
631 81), a function that is necessary for efficient viral DNA synthesis when these cellular proteins

cannot be targeted for proteasomal degradation by the virus-specific E3 ubiquitin ligase (24). Replication of the genome of a double mutant virus null for production of both the E1B 55 kDa and E4 Orf3 proteins proved to be so defective in HFFs that it was not possible to compare the sensitivity of its replication to exogenous IFN to that of the single mutant parents (data not shown). It will therefore be of considerable interest to determine whether the E1A or E4 Orf3 proteins allow repression of IFN-inducible genes when also made in HFFs stably producing the E1B 55 kDa protein.

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Table 1: Summary of effects of E1B 55 kDa protein substitution mutations on sensitivity of replication to IFN

Name	Substitution	Motif/Sequences target	Protein stability	IFN sensitivity
AdEasyE1Sub1[a]	C25G, E26A, T27A[a]	Surface exposed[b]	+[c]	-[e]
AdEasyE1Sub2[a]	R37G, P38A, P39A[a]	Surface exposed[b]	+[c]	-[e]
AdEasyE1Sub3[a]	E67A, S68A, R69A, P70A[a]	Surface exposed	+[c]	-[e]
AdEasyE1GSub4	L83A, L87A, L91A	Nuclear export signal	+[d]	-[e]
AdEasyE1GSub6	T211A, E212A, R214A, V215A	Surface exposed	ND	-[e]
AdEasyE1GSub7	V239E, R240A, F241A	p53 binding	-	+[f]
AdEasyE1Sub12	A284S, F285L	RNPI motif	+[d]	-[e]
AdEasyE1Sub13	R295A, P296A, K297A, S298A, R299A	RNPI motif	+[d]	-[e]
AdEasyE1GSub14	R240A	p53 binding	+[d]	-[e]
AdEasyE1Sub17	S490A, S491A	C-terminal phosphorylation sites, repression domain	+/-[d]	+/-
AdEasyE1Sub18	H373A	Surface exposed[b] putative $C_2 - H_2$ zinc finger	ND	-[e]
AdEasyE1Sub19	R443A, Y444A, D445A, E446A, T447A, R448A	Surface exposed[b]; repression domain	++[d]	+[f]

[a] The mutations that alter the E1B 55kDa-protein coding sequence did not to alter the overlapping coding sequence for the E1B 19 kDa protein.
[b] Predicted from hydropathy plots (Gonzalez *et al*, 2002).
[c] Based on comparison to wild-type of steady-state concentrations in cells transfected with pAdEasyE1 Shuttle vectors, and
[d] on comparison to wild-type in infected cells. + and ++ indicate as more stable as the wild type; - indicates decreased stability.
[e] Values of ratios of yields in presence and absence of 250 units/ml IFN within a factor of 2 of wild type values.
[f] Values of ratios of yields in presence and absence of 250 units.ml IFN ≤ 10-fold lower than that of wild type.
ND = not determined.

894

Figure 1: Inhibition of AdEasyE1Sub19 replication by exogenous IFN. HFFs were treated with IFN as described in Materials and Methods (IFN) or with vehicle only control (BSA), and infected with 50 p.f.u./cell AdEasyE1 (WT), AdEasyE1Δ2347 (ΔE1B) or AdEasyE1Sub19 (S19) for 36 hours (**A**), or with 3 p.f.u. each virus for 88 hours (**B**). In both cases, virus yields were determined by plaque assay on complementing 293 cells. The results represent the results of two independent experiments, and error bars indicate cumulative standard deviations.

Figure 2: Viral DNA synthesis is inhibited in AdEasyE1Sub19-infected cells treated with IFN. A. HFFs were infected with 30 p.f.u./cell AdEasyE1(WT), AdEasyE1Δ2347 (ΔE1B) or AdEasyE1Sub19 (S19) for the periods indicated, or mock-infected (M). The proteins listed at the right were examined by immunoblotting of total cell lysates as described in Materials and Methods. **B.** HFFs were infected with 30 p.f.u./cell AdEasyE1 (WT) or AdEasyE1Sub19 (S19) for 36 hours or mock infected (Mock), and the E1B 55 kDa protein visualized by immunofluorescence as described in Materials and Methods. **C.** HFFs were treated with interferon (IFN) or untreated (BSA) and infected with 3 p.f.u./cell of the virus indicated for 2 or 72 hrs. Viral DNA concentrations were measured by quantitative PCR, with cellular GADPH DNA as an internal control, as described in Materials and Methods, and are expressed relative to the input (2 hr.) concentrations. The value represents the average of two independent experiments, with cumulative standard deviations indicated by the error bars.

Figure 3: The Sub19 substitutions impair repression of expression of IFN-inducible genes by the E1B 55 kDa protein. HFFs, untreated (BSA) or treated with IFN (IFN) were infected with 200 p.f.u./cell AdEasyE1 (WT), AdEasyE1Δ2347 (ΔE1B) or AdEasyE1Sub19 (S19), and total cell RNA isolated 47 hrs. after infection. The concentrations of GBP1 (**A**) and IFIT2 (**B**) mRNAs were determined by quantitative PCR, after synthesis of cDNA by reverse transcription

from random primers, as described in Materials and Methods. The values shown represent the average and cumulative standard deviations (bars) of two independent experiments.

Figure 4: Stimulation of synthesis of primary transcripts of IFN-inducible genes in AdEasyE1Sub19-infected cells. Newly-synthesized RNA was isolated from HFFs infected with 50 p.f.u./cell AdEasyE1 (WT) or AdEasyE1Sub19 (S19), for 29 hrs., or mock-infected (M) and the concentrations of E1A, IFIT2 and IL6 pre-mRNAs and β-actin mRNA examined as described in Materials and Methods. The positions of DNA molecular mass markers (bp) are indicated at the left.

Figure 5: Effects of the E1B 55 kDa Sub19 alterations on activation of Stat1. Mock-infected HFFs were treated with 250 units/ml IFN or vehicle only control (BSA) for 24 hrs. Cells were infected with 200 p.f.u./cell AdEasyE1 (WT), AdEasyE1Δ2347(ΔE1B) or AdEasyE1Sub19 for the periods indicated, and the proteins listed at the right examined by immunoblotting of whole cell lysates as described in Materials and Methods. Stat1 phosphorylated on Tyr701 is designated Stat1-P.

Figure 6: The E1B 55 kDa protein cannot repress expression of IFN-inducible genes in uninfected cells. HFFs that stably express the E1B 55 kDa protein (E1B) or eGFP (GFP) coding sequences were isolated as described in Materials and Methods. **A.** The proteins listed at the right were examined by immunoblotting of total cell lysates. **B.** Cells were infected with 3 p.f.u./cell AdEasyE1 (WT) or AdEasyE1Δ2347 (ΔE1B), and the concentrations of viral DNA attained by 44 hrs. after infection relative to those at 2 hrs. p.i. determined by quantitative PCR as described in Materials and Methods. The values shown represent the results of two independent experiments, and bars the cumulative standard deviations. **C.** Cells were incubated

with medium containing 250 units/ml interferon (IFN) or vehicle only control (BSA) for 24 hrs., and the concentrations of GBP1 mRNA measured as described in the legend to Figure 3. The values shown indicate the average of two independent experiments, and bars the cumulative standard deviation. **D.** As panel C, except that concentration of IFIT2 mRNA were measured. **E.** The concentrations of BAX and MDM2 mRNAs were compared in HFF-GFP and HFF-E1B cells as described above and in Materials and Methods.

Figure 7: Replication of E1B 55 kDa null mutant genomes is not sensitive to IFN in A549 cells. A549 cells were treated with 1000 units/ml IFN (IFN) or vehicle only control (BSA) for 24 hrs. prior to and during infection with 0.5 p.f.u./cell AdEasyE1 (WT) or AdEasyE1Δ2347 (ΔE1B). Relative viral DNA concentrations at 46 hrs. after infection were determined as described in the legend to Figure 2. These values represent the average of two independent experiments, and the error bar the cumulative standard deviations.