Introduction

Subjugation of the intracellular environment by viruses is essential to ensure the effective expression and replication of the viral genome to allow production of progeny virions. One such viral strategy involves hijacking signalling pathways that ultimately control host gene transcription. Interactions between intracellular viral proteins and cellular kinases responsible for signal transduction are a means by which to achieve this. However, there is growing evidence to support the premise that glycoproteins on the surface of virus particles may trigger intracellular signalling pathways by interacting with their cognate receptors on the host cell membrane [1].

Binding of HSV-1 to permissive cells occurs through viral glycoproteins on the viral envelope interacting with specific receptors on the cell surface, triggering fusion of the plasma membrane with the outer envelope. Five of these glycoproteins are known to be involved in virion binding to the cell surface: gB, gC, gD and the heterodimer gH-L. Of the five, only gC is dispensable for producing productive infection as deletion of gB, gD or gH-L results in an entry-defective phenotype. While gH interacts with the αvβ3 integrin, with the paired immunoglobulin-like receptor, PILRα, acting as a receptor for gB [2][4][5].

One of the first studies to examine whether HSV-1 glycoproteins play a role in the induction of signalling found that gD was able to block Fas-mediated apoptosis [6]. U937 monocytic cells were rendered resistant to apoptosis after infection with UV-inactivated HSV virions, when co-cultured with a stably transfected HSV-1 gD-expressing cell line or after treatment with soluble gD. Inhibition of NF-κB signalling by the introduction of the dominant-negative NF-κB repressor abolished protection from gD-induced Fas-mediated apoptosis. More specifically, the interaction of gD with one of the gD receptors, HVEM, was involved in preventing apoptosis induction [1].

There are reports showing that HSV-1 triggered the translocation of NF-κB by six hours post-infection [7]. However, it has come to light that HSV-1 may induce two distinct phases of NF-κB activity. The initial phase is transient, lasting only two hours, and occurred shortly after viral adsorption by both wild-type and UV-inactivated virus particles. This correlates with evidence showing that supernatant taken from gD-expressing cells may also cause an increase in NF-κB binding activity in monocytoid cells by 30 minutes post-treatment [6]. A second phase relied on de novo viral protein synthesis as it was stimulated only by replication-competent HSV-1 and not by UV-inactivated virions [8]. During this second phase, NF-κB complexes were shown to have associated with their consensus sequence found in the promoter region that drives ICP0 expression [8].

Abstract

The envelope of HSV-1 contains a number of glycoproteins, four of which are essential for virus entry. Virus particles lacking gB, gD, gH or gL are entry-defective, although these viruses retain the ability to bind to the plasma membrane via the remaining glycoproteins. Soluble forms of gD have been shown to trigger the nuclear translocation of the NF-κB transcriptional complex in addition to stimulating the production of Type I interferon. By taking advantage of the entry-defective phenotype of glycoprotein-deficient HSV-1 virus particles, the results presented here show that binding of virions to cellular receptors on the plasma membrane is sufficient to stimulate a change in cellular gene expression. Preliminary microarray studies, validated by quantitative real-time PCR, identified the differential expression of cellular genes associated with the NF-κB, PI3K/Akt, Jak/Stat and related Jak/Src pathways by virions lacking gB or gH but not gD. Gene induction occurred at a few particles per cell, corresponding to physiological conditions during primary infection. Reporter assay studies determined that NF-κB transcriptional activity is stimulated within an hour of HSV-1 binding, peaks between two and three hours post-binding and declines to background levels by five hours after induction. The immediate, transient nature of these signalling events suggests that HSV-1 glycoproteins, particularly gD, may alter the cellular environment pre-entry so as to condition the cell for viral replication.
In comparison to wild-type gD, a mutated form of gD that was unable to bind to HVEM did not stimulate NF-κB activity in cocultured monocytes [9]. HEp-2 cells, which do not express HVEM, lacked detectable NF-κB signalling after infection with UV-inactivated HSV-1, implying that gD-mediated NF-κB stimulation may be dependent on expression of HVEM [9].

Soluble glycoproteins from beta- and gammaherpesviruses can also stimulate intracellular signalling pathways; gB of CMV has been shown to stimulate the differential expression of cellular transcription factors and interferon-stimulated genes (ISGs), and soluble gp350 of EBV can trigger NF-κB activation [10] [11][12].

The treatment of cells with soluble glycoproteins or UV-inactivated virus has provided strong evidence that HSV-1 binding to the plasma membrane triggers signal transduction in the host cell as a prelude to infection, but interpretation of these experiments is not straightforward. The use of soluble glycoproteins does not mimic the binding of individual virus particles and the physiological significance of the results is difficult to assess. The use of UV-inactivated particles in signalling studies simulates infection but these particles are able to enter cells and deliver virion proteins to the cytoplasm. Experiments with UV-inactivated particles do not therefore distinguish between events arising from virus binding from those resulting from virus entry.

The main focus in these studies was to examine signalling events using virus particles that are capable of binding to the cell surface but are incapable of entry due to the absence of one of the three essential glycoproteins, gB, gD or gH [13][14][15][16], and to find whether signalling occurs at particle concentrations that would mirror physiological conditions.

Materials and Methods

Viruses and Cells

All cells other than Human Foreskin Fibroblasts (HFF) (ATCC cell line CRL-2522) were grown in Glasgow Modified Eagles Medium supplemented with 10% foetal bovine serum, 4 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. HFF cells were grown in Dulbecco modified Eagles medium supplemented as described above but with the addition of 1x MEM non-essential amino acids.

Working stocks of HSV-1 SC16 were grown on HaCaT cells and assayed on Vero cells [17]. Mutant viruses lacking functional genes for gH (SCgHZ) [14], gB (HFEMdUL27-lacZ) [18] or gD (SC16gDdelZ) [18] were grown and assayed on helper cell lines expressing the corresponding glycoprotein: CR1 cells, expressing gH [19]; D6 cells expressing gB [13]; and VD60 cells expressing (SC16gDdelZ) [18] were grown and assayed on helper cell lines.

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Purified preparations of WT virus, or of virions lacking individual glycoproteins, were produced as previously described [20]. Purified preparations were assayed for particle numbers using electron microscopy [21] and for infectivity by plaque assay on Vero cells or, in the case of glycoprotein-deficient virions, on the relevant helper cell line. Preparations of WT virions had a particle to infectivity ratio of 3×10^6. Preparations of gH-negative, gD-negative and gB negative virions had particle to infectivity ratios of 2×10^6, 5×10^6 and 3×10^6 respectively. The maximum virion dose of entry-incompetent virions used in the experiments described in this paper was 1000 particles per cell and it therefore follows that in the maximum infectivity of a preparation of an entry-defective virus (i.e. using gB-negative virions) would be less than one cell per thousand. Virus preparations were deglycosylated with 500 U of PNGase F for 18 hrs at 37 °C in the absence of denaturing buffer [22]. Removal of N-linked sugars was confirmed by a shift in the electrophoretic mobility of gD on a protein denaturing gel.

Signalling Experiments

Human foreskin fibroblast cells (HFF) were seeded at 1.5×10^6 in a 175 cm² flask and grown to 90% confluence then growth-arrested in medium supplemented with only 100 U/ml Penicillin-G and 100 μg/ml Streptomycin for five days. Real-time PCR experiments were conducted with growth-arrested HFFs that were inoculated in triplicate with 1000 particles/cell of entry-defective HSV-1 in warm, serum-free medium, with parallel mock-infected cells that received an equivalent volume of PBS in warm serum-free medium. Inoculations took place at 37 °C. At the indicated times after the addition of virus, the medium was removed, the cells were lysed with TRI Reagent (Sigma) and total RNA was immediately purified. For each entry-defective HSV-1 mutant, three independent biological replicates were carried out. Microarray experiments were conducted under the same conditions using wild-type HSV-1 (SC16) at an MOI of 20.

RNA Purification and cDNA Preparation

Total RNA was isolated from TRI reagent lysates as per the manufacturer’s instructions. Contaminating genomic DNA was digested with 2 U DNase I (Invitrogen) and RNA was re-purified with TRI reagent. cDNA was derived from 30 μg total RNA using anchored oligo (dT)20 primers (Invitrogen) as described in the manufacturer’s instructions. For each entry-defective HSV-1 mutant, the cDNA from three independent infections were used in technical triplicates for real-time PCR reactions.

Signalling-Specific Microarray

Microarray analysis was carried out using the Signal Transduction PathwayFinder cDNA array as per the manufacturer’s instructions (SuperArray). Nylon cDNA microarrays membranes were pre-hybridised with supplied sheared salmon sperm DNA at 60 °C for one to two hours. The pre-hybridisation solution was replaced with the biotin-dUTP labelled cDNA probe, which was diluted in sheared salmon sperm DNA. The solution was left to hybridise at 60 °C overnight. The following morning the cDNA probe was discarded and the membrane was washed twice then blocked in Solution Q, as provided by the manufacturer. After 40 minutes, the blocking solution was discarded and the membrane was incubated with a binding buffer, Solution F. After washing, the chemiluminescent substrate was incubated with the membrane for two to five minutes at room temperature. The microarray membrane was then exposed to X-ray film. Analysis was performed using GEArray Expression Analysis Suite as provided by the manufacturer (Superarray). All the data sets used in the microarray analysis are available in Table S1.

Real-Time PCR

cDNA derived from cells that were mock-infected or inoculated with 1000 particles/cell of entry-defective ΔgB, ΔgD and ΔgH virions was used for real-time PCR analysis. Primers were designed, using the Primer3 software [23], to anneal at 60 °C, with a minimum product melting temperature of 80 °C (Table S2). Reactions were set-up with cDNA corresponding to 100 ng total RNA, primers (70 nM), and 10 μl SYBR Green PCR master mix (Agcene) made to a total volume of 20 μl with nuclease-free dH2O. A Corbett Rotorgene 3000 was used to determine the levels of SYBR green fluorescence over 40 cycles. Samples were denatured for 10 minutes at 95 °C, annealed at 60 °C for 30 seconds, with extension of the primer product at 72 °C for 40
seconds then a final 80°C step for 20 seconds that removes background fluorescence from primer-dimers. A melt curve analysis was produced at the end of the cycling to ensure the specificity of PCR product amplification and associated SYBR green fluorescence. Relative gene expression levels between mock-inoculated and inoculated cells were calculated using the Pfaffl method with potential variations in cDNA quantity between samples normalised to the transcript for ribosomal protein L13a (RPL13a) [24]. Serial dilutions of PCR product across 8 logs were used to determine the efficiency of PCR amplification for each primer set under the above conditions so that the relative quantitation could be adjusted as defined by the Pfaffl method.

A relative change in expression of two-fold was set as a threshold for determining whether differential expression of a gene had occurred. The p values associated with the fold-change in expression were calculated using a Students t-test on the real-time PCR C T values comparing triplicate mock-inoculated and HSV inoculated cells.

Luciferase Assays
An NF-kB reporter assay system was designed using a pGL4-NF-kB construct (Promega). Quadruplicate repeats of the NF-kB binding consensus sequence 5′-GGGAATTTCC-3′ were excised from a p-NF-kB-Luc (Gift from Dr. Heike Laman, University of Cambridge) vector using KpnI and HindIII and ligated into the pGL4.20 vector multiple cloning site using the same restriction sites. pGL4.20 was chosen as it is optimized for more efficient expression in mammalian cells, compared to previous generations of luciferase constructs, and contains fewer transcription factor consensus sequences such that background luciferase expression is reduced. HFFs were electroporated in batches of 6 with 105 cells per well of a 24-well plate. After 48-hours recovery in supplemented media, the transfected HFFs were serum-starved for 3 h before 24-hour exposure to 1000 particles per cell of virions lacking either gD, gB or gH and RNA was extracted for cDNA synthesis immediately (0 h) or after 6 h. Table 2 gives the results of two independent experiments for those genes that were differentially expressed between 0 and 6 h post treatment and is compared to the initial microarray results in Table 3. It is striking that in virtually every instance where gene expression is modified by virion binding, gD appears to be the key effector. Virions lacking gH or gB had were capable of altering cellular gene expression while those lacking gD had no significant effect. The results can be summarised as follows.

JAK/STAT and JAK/Src Pathways
The JAK/STAT and related JAK/Src pathway show a similar response to that seen for NF-kB. Of the JAK/STAT-responsive genes, only NOS2A is regulated by more than one pathway, with AgB and AgH virions inducing their transcription and AgD particles having no significant effect (Table 2). Although the JAK/Src pathway has only two targets on the signalling microarrays - upon which the real-time PCR experiments were based - BCL2L1 is unique to the pathway, with BCL2 expression regulated by multiple signalling routes. Interestingly, while AgB and AgH virions up-regulated BCL2 and BCL2L1, AgD virus particles appeared to down-regulate both targets by a significant amount.

PI3K/Akt Pathway
Induction of this pathway may be responsible for the upregulation of CCND1, MYC, BCL2 and MMP7 after inoculation with AgB and AgH but not AgD HSV-1 (Table 2). Differential expression of CCND1 and MYC can be regulated by the Wnt and PI3K signalling pathways, with MYC also being controlled by Protein Kinase C. The up-regulation of MMP7, which is the only target unique to the PI3K pathway that is differentially expressed, highlights the potential crosstalk between signalling pathways and cannot exclude the involvement of PI3 kinase signalling as a pathway leading to transcription of all four cellular genes.

NF-kB Pathway
Both AgB and AgH virions are capable of stimulating an NF-kB response (Table 2), a result that correlates with the preliminary microarray data (Table 1). Transcripts for both BIRC-2 and BIRC-3 are increased above the two-fold threshold after inoculation with AgD HSV-1 but not AgD. There appears to be no change in BIRC-1 expression after inoculation with any of the three entry-defective mutants. Components of the NF-kB transcriptional complex, NFKB1 and REL, showed similar levels of increased transcription after binding by AgB and AgH virions, a response that was absent in cells stimulated with gD-deficient virions. Two markers of inflammation, CCL2 and PECAM1, also showed a significant change in gene expression at 6 hpi with entry-defective HSV-1 lacking gB or gH but not gD.

The downstream signalling appears specific as not all NF-kB targets were up-regulated. The NF-kB repressors, NFKBIA and NFKBIB, were only up-regulated by AgB virion binding (Table 2).

Kinetics of NF-kB Activation
To confirm that virion binding induces NF-kB and to examine the kinetics, an NF-kB promoter-driven luciferase construct was
**Table 1.** Fold change in gene expression after inoculation with entry-defective HSV-1.

| Gene Description | ΔgB | ΔgD | ΔgH |
|------------------|-----|-----|-----|
| **NF-KB** | | | |
| BCL2A1 hematopoietic BCL2-related protein A1 | −3.85 * | 5.56 | 1.42 |
| BIRC1 baculoviral IAP repeat-containing 1 | −2.33 | −1.12 | −3.13 ** |
| BIRC2 baculoviral IAP repeat-containing 2 | 3.85 | 3.98 | 1.72 |
| BIRC3 baculoviral IAP repeat-containing 3 | 2.88 | 1.00 | 7.71 *** |
| CCL2 macrophage chemotactant protein-1 | 2.32 * | 1.34 | 2.13 * |
| LTA lymphotoxin alpha (TNF superfamily, member 1) | −1.22 | 2.31 | −1.20 |
| NFKB1 nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105) | 2.98 * | −1.92 | 3.09 ** |
| NFKBIA nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha | −1.19 | 1.23 | 2.54 ** |
| PECAM1 platelet/endothelial cell adhesion molecule (CD31 antigen) | 4.60 * | 1.00 | 3.09 * |
| **JAK/STAT** | | | |
| A2M alpha-2-macroglobulin | 5.07 * | 1.79 | 2.23 |
| CSN2 casein beta | 5.23 ** | −1.54 | 1.48 |
| CXCL9 chemokine (C-X-C motif) ligand 9 | 2.22 | −1.59 | 1.06 |
| IRF1 interferon regulatory factor-1 | 1.90 | −2.94 | 1.00 |
| MMP10 matrix metalloproteinase 10 | 1.09 | −1.12 | 2.50 |
| NOS2A nitric oxide synthase 2A | 1.26 | 1.21 | 8.01 ** |
| **PI3K/AKT** | | | |
| BCL2 B-cell CLL/lymphoma 2 | 3.54 | −1.54 | 1.86 ** |
| CCND1 cyclin D1 | 1.54 | −9.09 *** | 3.22 * |
| FN1 fibronectin 1 | 1.50 | 1.03 | 6.28 ** |
| MMP7 matrix metalloproteinase 7 | 2.05 | 1.91 | 3.05 ** |
| MYC v-MYC myelocytomatosis viral oncogene homolog | 2.83 | 1.09 | 4.55 |
| WISP2 WNT1 inducible signaling pathway protein 2 | −7.69 ** | 1.50 | −4.55 *** |
| **JAK/Src** | | | |
| BCL2 B-cell CLL/lymphoma 2 | 3.54 | −1.54 | 1.86 ** |
| BCL2L1 BCL2-like 1; Bcl-X | −4.92 | −5.00 ** | −3.33 |
| **p53** | | | |
| GADD45A growth arrest and DNA-damage-inducible, alpha | 5.22 * | −1.15 | 1.96 |
| IGBP3 insulin-like growth factor binding protein 3 | −6.67 * | −1.04 | 1.18 * |
| MDM2 Mdm2 p53 binding protein homolog (mouse) | 6.83 * | −1.82 | 2.89 |
| **Phospholipase C** | | | |
| EGR1 early growth response 1 | −2.70 | −2.50 * | 1.09 |
| FAS fas (TNF receptor superfamily, member 6) | −2.38 | 1.15 | −2.63 * |
| FOS FBV murine osteosarcoma viral oncogene homolog | −2.78 * | 1.56 | −1.59 |
| JUNB jun B proto-oncogene | −1.01 | −2.56 | −1.33 |
| PTGS2 rostaglandin-endoperoxide synthase 2 | 1.26 | 1.21 | 8.01 |
| **TGF-β** | | | |
| CDKN1A cyclin-dependent kinase inhibitor 1A | 1.12 * | 4.01 * | 1.10 |
| CDKN2A cyclin-dependent kinase inhibitor 2A | 1.89 | 1.35 | −4.17 ** |
| CDKN2B cyclin-dependent kinase inhibitor 2B | −1.19 | 2.72 | −2.27 |
| CDKN2D cyclin-dependent kinase inhibitor 2D | 1.31 | −4.55 ** | 1.05 |
| **Retinoic Acid** | | | |
| CDX1 caudal type homeobox 1 | 1.31 | 2.05 | 1.09 |
| CTS5 cathepsin D | −1.05 | −3.70 | −1.12 |
| EN1 engrailed homeobox 1 | −1.10 | −2.22 | 1.00 |
| RBP1 retinol binding protein 1, cellular | 1.68 | −3.03 * | 1.00 |
| **Androgen** | | | |
| CDK2 cyclin-dependent kinase 2 | 3.86 | −1.45 | 3.44 ** |
| KLK2 kallikrein-related peptidase 2 | 6.83 *** | −1.82 | 2.89 |
transfected into HFF cells and cells were treated with ΔgH virions (1000 particles per cell) or were mock-treated. Cells were harvested every hour until 9 hr post treatment, lysed and assayed for luciferase activity in triplicate. Figure 1(a) shows the fold-change in luciferase activity of those HFFs inoculated with ΔgH in comparison to mock-inoculated cells at the same time point. Biological duplicates of the inoculations were performed with the lysates used in triplicate reporter assays. At 1 hpi, there is an average of 2.4 fold change in luciferase activity from the NF-κB reporter, rising to 3.5 at 2 hpi and is maintained through to 3 hpi whereby it drops to 1.8 at 4 hpi, which correlates with the half-life of luciferase [28]. By 5 hpi, and until 9 hpi, luciferase activity drops to baseline levels at, or near, 1-fold change. Error bars represent the standard error across the six readings taken from technical triplicates on biological duplicates.

As induction of the NF-κB reporter construct occurred within one hour of inoculation with ΔgH virions and peaked at around two-and-a-half hours post-inoculation, then the transcripts previ-
uously shown in Table 2 to be up-regulated as a result of ΔgH virions binding were examined for changes in transcript levels at two-and-a-half hours after inoculation with ΔgH. Figure 1(b) compares expression levels at two-and-a-half and six hours post-inoculation. Transcripts for the NF-κB-responsive chemokine CCL2 were up-regulated to a greater degree at two-and-a-half hours than at six hours post-inoculation. Of the NF-κB-responsive genes, REL transcription was induced to a higher level whereas PECAM1 transcript abundance was reduced in comparison to six hours. Although JAK/STAT-responsive targets were not differentially expressed by two-and-a-half hours, the JAK/Src targets REL and BCL2 and BCL2L1 were up-regulated above the two-fold arbitrary threshold to similar transcript levels that were seen at six hours post-inoculation.

Interferogenic Capacity of Entry-Defective Virions

The initial microarray screen was uninformative with respect to interferon signalling, but this was of interest because soluble gD has been reported to induce IFN [29], while, in contrast, ISG induction is reported to require virion entry but not de novo protein synthesis [30]. Primers were designed to Type I interferons, α (IFNA1) and β (IFNB1), and the newly designated Type III interferon λ (H/29). Human fibroblasts do not produce interferon gamma. Additionally, the cDNA preparation was used to identify any change in the abundance of ISGs. Both ΔgB and ΔgH virions were able to stimulate the up-regulation of IFN-α expression 2.81 fold and 2.34 fold, respectively, with a high degree of statistical significance (p≤0.05) (Table 4). HSV-1 particles lacking gD were unable to do so, with a fold change of −1.11 and a p = 0.15 (Table 4).

IFN-α transcript levels were up-regulated 2.22 fold by ΔgB virions and 2.06 fold (p = 0.01) by ΔgD virions, again with a high degree of statistical significance. Inoculation with virus particles lacking gH led to a −1.07 fold change (p = 0.44) in IFN-α expression when compared to mock-infected cultures. All three entry-defective mutants were unable to modulate the expression of IFN-λ above the arbitrary two-fold threshold.

The glycosylation status of IFN-inducing viral glycoproteins is a potential determinant of induction efficacy [22][31]. HSV-1 gD has three potential N-glycosylation sites but lacks O-linked glycosylation. ΔgH virions, that retain expression of gD, were treated with the endoglycosidase, PNGase F, which removes N-linked sugars. A long incubation was required as the denaturing buffer was excluded from the reaction to avoid conformational changes that might abrogate virion binding. As previously shown, wild-type virions treated in a similar manner retained infectivity (Figure S1) [22].

ΔgH virions that had been incubated for 18 hrs in the presence or absence of PNGase. Glycosylated and deglycosylated ΔgH virions stimulated a 2.23 and 2.28 fold increase in IFN-α transcription, respectively. These results suggest that glycosylation has no impact on the interferogenic properties of ΔgH virions, unlike the glycoproteins of MHV and TGEV, which appear to stimulate Type I IFN through

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| Table 3. Comparison of microarray with real-time PCR data for differentially expressed genes. |

| Gene   | ΔgB Microarray | ΔgB Real-Time | ΔgD Microarray | ΔgD Real-Time | ΔgH Microarray | ΔgH Real-Time |
|--------|---------------|---------------|---------------|---------------|---------------|---------------|
| NF-κB  | BIRC2         | 3.66          | 3.85          | 1.20          | 3.98          | 2.92          | −1.01         |
|        | BIRC3         | 4.45          | 2.88          | 1.27          | 1.00          | 3.71          | 7.71          |
|        | CCL2          | 2.09          | 2.32          | −1.51         | 1.34          | 3.16          | 2.13          |
|        | NFKB1         | 3.54          | 2.98          | 1.13          | −1.92         | 2.61          | 3.09          |
|        | NFKBIA        | 2.35          | −1.19         | 1.07          | 1.23          | 1.48          | 2.54          |
|        | NFKBIB        | 2.72          | -             | 1.16          | -             | 1.40          | -             |
|        | PECAM1        | 3.49          | 4.60          | 1.22          | 1.00          | 4.44          | 3.09          |
|        | REL           | 2.05          | -             | −1.25         | -             | 2.63          | -             |
| JAK/STAT| A2M           | 3.54          | 5.07          | 1.77          | 1.79          | 2.55          | 2.23          |
|        | MMP10         | 2.87          | 1.09          | 1.52          | −1.12         | 2.62          | 2.50          |
|        | NOS2A         | 5.00          | 1.26          | −1.09         | 1.21          | 4.41          | 8.01          |
| PI3K/AKT| BCL2          | 2.86          | 1.86          | −2.33         | −1.54         | 2.13          | 1.86          |
|        | MMP7          | 3.67          | 2.05          | −1.03         | 1.91          | 2.04          | 3.05          |
|        | MYC           | 4.53          | 2.83          | −1.04         | 2.85          | 2.67          | 2.48          |
| Jak/Src | BCL2          | 2.86          | 1.86          | −2.33         | −1.54         | 2.13          | 1.86          |
|        | BCL2L1        | 2.56          | 1.42          | −1.92         | −5.00         | 2.09          | −1.18         |
| Androgen| CDK2          | 3.01          | 3.86          | 1.18          | −1.45         | 3.08          | 3.44          |

There was a degree of correlation between the change in expression determined by microarray studies and those confirmed by real-time PCR, particularly for genes under the control of NF-κB. Note: rel and nfkbib were not present on the original signalling-specific microarray, but were included in the real-time analysis, as they are known to be up-regulated as part of the NF-κB feedback mechanism.

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Figure 1. Kinetics of signalling activation. (A) An NF-κB luciferase construct was transfected into HFF, which were left to serum-starve for five days. Cells were then mock-infected or inoculated with 1000 particles/cell of entry-defective ΔgH HSV-1 then lysed at the indicated times and assayed for luciferase activity. Error bars are the ±/− standard error across triplicate luciferase assays for each biological duplicate. (B) NF-κB, JAK/STAT, JAK/Src and PI3K/Akt-responsive genes that were previously shown to be differentially expressed at 6 hours post-inoculation with ΔgB and ΔgH virions were examined for changes in expression at two-and-a-half hours after inoculation with 1000 particles/cell of ΔgH virions. The induction of JAK/STAT and PI3K/ Akt targets is not significantly up-regulated above the two-fold threshold by two-and-a-half hours post-inoculation. A number of NF-κB-associated transcripts are significantly up-regulated, with ccl2 showing a far greater induction at two-and-a-half hours than six hours. Both JAK/Src targets, bcl2 and bcl2l1 are induced to similar levels found at six hours post-inoculation. Error bars are the ±/− standard error across triplicate biological replicates.

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These cultures exhibited no induction of IFN or ISGs (Table 4).

To ensure that these observations could not result from a background of infection by competent virus, parallel cultures were infected with wild-type HSV-1 at an MOI of 0.01, a level of infection some tenfold higher than would result from the ‘worst case’ inoculation with 1, 10, 100 or 1000 particles per cell of entry-defective HSV-1. Changes in gene transcripts after infection with 1 particle/cell compared to mock-infected cells were not stimulated (data not shown). These results are compatible with our model, shown in Fig. 3.

Effects of ΔgH Virion Multiplicity on Stimulating Signalling

All the experimental work described above was performed using treatments with 1000 virus particles per cell, a condition that would be unlikely to persist in natural primary infections. To establish whether much lower virion numbers would be effective, HFFs were serum-starved for five days then mock-inoculated or inoculated with 1, 10, 100 or 1000 particles per cell of ΔgH HSV-1 for six hours. Infections were carried out in duplicate with mock-inoculated controls that received an equivalent volume of PBS. Total RNA was purified and reverse transcribed, with the cDNA from each duplicate inoculation used in triplicate for real-time PCR.

The NF-κB responsive genes NFKB1, REL and CCL2, in addition to the JAK/STAT target NOS2A, were used as they had previously been shown to be differentially expressed after inoculation with 1000 particles/cell of ΔgH HSV-1. Recalling that the more abundant a transcript the fewer number of cycles it takes to cross a threshold point in the exponential phase of PCR product accumulation (the Ct value), Figure 2 plots the ΔCt value on the y-axis, (i.e. the Ct value for the gene of interest after normalisation against the RPL13) against the multiplicity of infection on the x-axis. As the multiplicity of ΔgH increases the Ct value decreases, indicating an increase in the abundance of transcripts for all three NF-κB targets (Figure 2(a)). A logarithmic regression analysis across three logs of multiplicity show a high correlation between the four Ct values for each transcript with r2 >0.98. Similarly for the JAK/STAT responsive target NOS2A there are almost two cycles of a difference between the level of transcripts after infection with 1 particle/cell compared to 1000 particles/cell (Figure 2(b)). This larger response than those for NF-κB responsive genes is indicative of the different level of induction shown with previous real-time PCR analysis of these transcripts (Table 2).

Arrows in Figure 2 indicate the Ct value for each of the four targets examined. Inoculation with 1 particle cell gave no detectable increase in transcript abundance but an increase was observed using 10 particles per cell and the dose response curves strongly suggest that lower doses have an effect. Interpreting these data in the context of infection is not straightforward as particle:infectivity ratios for herpes simplex virus are, at best, 20 or more. On this basis a single infectious unit per cell is clearly sufficient to increase transcript abundance, indicating that signalling occurs at ‘physiological’ multiplicities of infection.

Discussion

Evidence from previous studies using soluble HSV-1 glycoprotein and UV-inactivated virus suggested that binding of HSV-1 virions to the cell surface might be sufficient to stimulate intracellular signalling pathways. We undertook preliminary microarray studies with entry-defective HSV-1 virions and identified a number of pathways that were stimulated at early time points during infection.

Microarray experiments are inevitably vulnerable to false positives and negatives due to non-specific binding of labelled cDNA probes, necessitating a more robust follow-up with highly sensitive methods. Due to such confounders, these data were used exclusively as a guide to select, for real-time PCR, the transcriptional targets of intracellular signalling pathways stimulated after inoculation with entry-defective HSV-1.

Gene targets associated with the NF-κB, JAK/STAT/Src, and PI3K/Akt pathways were shown to be differentially expressed after inoculation with glycoprotein-deficient virions, with the majority of signalling events being associated with the presence of gD on the envelope. Nineteen other signalling pathways present on the preliminary microarray experiments, and confirmed by real-time PCR, were not stimulated (data not shown). These results are compatible with our model, shown in Fig. 3.

Real-time PCR confirmed that changes in transcription associated with the NF-κB, JAK/STAT, JAK/Src and PI3K pathways were modulated as a result of virion binding, all of which required gD on the envelope surface. To demonstrate that signalling occurred at physiologically relevant multiplicities of infection, HFFs were inoculated with either 1000, 100, 10 or 1 particle/cell of entry-defective HSV-1. Changes in gene transcription occurred in a dose-dependent manner and were detectable at 10 virus particles per cell. Given the particle:infectivity ratios usually quoted for HSV1, we argue that this corresponds to physiological conditions (i.e. less than one infectious unit per cell), but it is impossible to know the circumstances that pertain in vivo when a single cell becomes infected. What seems almost certain is that an infected cell will normally present an uninfected neighbouring cell with 10 or more progeny particles.

HSV-1, as well as beta- and gammaherpesviruses, are capable of stimulating the NF-κB pathway in a bi-phasic manner, with our

Table 4. Interferon response to HSV-1 virion binding.

| Name               | Description                             | ΔgB | ΔgD | ΔgH | MOI 0.01 |
|--------------------|-----------------------------------------|-----|-----|-----|----------|
| IFNA1              | interferon, alpha 1                     | 2.63* | -1.11 | 2.25 * | -1.23   |
| IFNB1              | interferon, beta 1, fibroblast          | 3.01* | 2.15 * | -1.07 | -1.18   |
| IL29               | interleukin 29 (interferon, lambda 1) | 1.51 | -1.33 | -1.69 | 1.11    |
| ISG54              | IFN-induced protein 54                  | 1.20 | 1.57 | -1.54 | 1.45    |
| IRF1               | interferon regulatory factor 1          | 1.21 | 1.07 | 1.36 | 1.00    |
| IRF3               | interferon regulatory factor 3          | 1.14 | -1.10 | 1.05 | 1.08    |
| IRF7               | interferon regulatory factor 7          | 1.19 | 1.03 | -1.05 | -1.23   |
| IRF9               | interferon regulatory factor 9          | -1.23 | 1.03 | 1.44 | -1.33   |

Note. *p < 0.05.

Binding by ΔgB and ΔgH virions up-regulated the expression of IFN-α whereas ΔgB and ΔgD virions were able to stimulate an increase in IFN-β. Infection with wild-type HSV-1 at an MOI of 0.01 was insufficient to cause similar increases in interferon expression. Interferon-stimulated genes were not up-regulated above the two-fold threshold by binding of any of the entry-defective HSV-1 mutants. Wild-type infection at a low MOI was also insufficient to cause in increase in ISG transcripts when compared to mock-infected cells.

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a lectin-like action, HSV-1 gD may trigger IFN up-regulation through a different mechanism, possibly analogous to that used to stimulate other signalling pathways.

Five interferon-stimulated genes were chosen to examine whether they were differentially expressed by virion binding. IFN-induced protein 54 (ISG54) transcripts are routinely used as a marker of ISG induction. Binding by ΔgB, ΔgD or ΔgH virions was insufficient to cause changes in the transcript abundance of interferon regulatory factor-1, -3, -7, -9 or ISG54 (Table 4).

To ensure that these observations could not result from a background of infection by competent virus, parallel cultures were infected with wild type virus at an MOI of 0.01, a level of infection some tenfold higher than would result from the ‘worst case’ scenario using preparations of entry defective virions (see Methods). These cultures exhibited no induction of IFN or ISGs (Table 4).

Signaling by HSV Glycoproteins
Figure 2. Effects of virion multiplicity on gene transcription. Low multiplicities of infection, which may represent physiological conditions, were sufficient to trigger an intracellular signalling response. (A) As the number of inoculated ΔgH particles increases, the number of cycles taken to reach the cycle threshold decreases for the NF-KB-responsive genes nfkb1, ccl2 and rel, indicating an increased abundance of transcript. (B) A similar dose response is seen for the JAK/STAT target nos2a. Error bars are the +/- standard error across triplicate biological replicates.

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data supporting that an early, transient induction is reliant on virions expressing gD [8]. Suppression of NF-κB activity is via negative feedback up-regulation of the inhibitor IκBa, which was also stimulated by the binding of entry-defective HSV-1 virions. The triggering of early NF-κB transcriptional activity was most likely through the coupling of gD on entry-defective virions to the TNF superfamily receptor HvEM [1]. In doing so, not only does the initial activation of this pathway allow for the subsequent sequestration of the NF-κB p65 subunit to the ICP0 promoter, but is crucial for immediate-early gene transcription and subsequent HSV-1 replication [8].

Intracellular signalling induced by soluble gD can protect against Fas-mediated apoptosis with inhibition of NF-κB activity is via negative feedback up-regulation of the inhibitor IκBa, which was also stimulated by the binding of entry-defective HSV-1 virions. The triggering of early NF-κB transcriptional activity was most likely through the coupling of gD on entry-defective virions to the TNF superfamily receptor HvEM [1]. In doing so, not only does the initial activation of this pathway allow for the subsequent sequestration of the NF-κB p65 subunit to the ICP0 promoter, but is crucial for immediate-early gene transcription and subsequent HSV-1 replication [8].

Intracellular signalling induced by soluble gD can protect against Fas-mediated apoptosis with inhibition of NF-κB signalling leading to a loss of this protection [6]. Infection with UV-inactivated virions also led to an increase in the expression of the anti-apoptotic protein c-IAP2 (birc3), which we have demonstrated to be up-regulated after inoculation with entry-defective virions containing gD. Additional studies have supported the anti-apoptotic role for NF-κB during HSV-1 infection yet there are conflicting data that demonstrate possible pro-apoptotic activity [34][35]. This inconsistency may be due to differing cell types used in those studies. Primary human foreskin fibroblasts have been shown to be resistant to apoptosis after infection with recombinant HSV-1 that is unable to express ICP4 or ICP27 whereas infection with either virus has been shown to cause apoptosis in transformed cell lines [36].

Aspects of the HSV-1 life cycle, such as stimulating the progression of the cell cycle in the absence of serum, may be sufficient to induce a stress response and trigger apoptosis. Both bcl2 (Bcl-2) and bcl2l1 (Bcl-xl) belong to the Bcl-2 family of apoptosis regulators that provide cellular protection from a range of harmful stimuli such as cytokine deprivation, UV- and γ-irradiation [37]. Bcl-2 and Bcl-xl are found in the outer mitochondrial membrane and are thought to suppress apoptosis by blocking mitochondrial outer-membrane permeabilisation through the sequestration of pro-apoptotic Bcl2 family members [38]. Given the up-regulation of four anti-apoptotic genes, birc2, birc3, bcl2 and bcl2l1, through the activation of multiple signalling pathways by entry-defective HSV-1, this establishes a role for gD binding in shifting the intracellular environment towards a more anti-apoptotic stance.

**Figure 3. Model for glycoprotein-receptor interactions in the induction of intracellular signalling pathways by HSV-1.** Glycoprotein D acts as the main signalling molecule on the surface of the HSV-1 envelope. gH interacts with αvβ3 integrins to potentially trigger the production of IFN-β, which is known to involve IRF-3 and 7 [48]. Binding by gD to HvEM may lead to the activation of TRAF molecules, which in turn stimulate the NF-κB signaling cascade. This pathway up-regulates a number of cellular genes in addition to augmenting early viral gene expression. NF-κB-responsive genes, birc2 and birc3, have an anti-apoptotic role, but paradoxically, inflammatory mediators such as ccl2 are also up-regulated. gD-induced signalling of the Jak/Stat and Jak/Src pathways also results in the differential expression of genes associated with anti-apoptosis and inflammation. The up-regulation of c-Myc could lead to a corresponding increase in cdk2, which has a role in promoting DNA replication and gene transcription during infection. It should be noted that most signalling cascades have been elucidated in non-fibroblast cells lines, so the role of specific kinases may vary in HFFs.

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It is less apparent as to the biological relevance of an innate immune response stimulated through HSV-1 binding. It may be that there is a "cost" associated with altering the intracellular environment, which leads to the differential expression of cytokines, such as ccl2, that are under similar transcriptional regulation as those host factors that are favourable for virus replication.

Signalling by secreted Type I IFNs occurs through the Jak/Stat pathway results in the expression of various ISGs; a response that is also triggered by virus entry [39]. Nevertheless, productive infection with HSV-1 can down-regulate the triggered ISG response, allowing viral replication to continue unhindered [40]. Despite our evidence that gD binding by entry-defective virions can induce IFN-γ mRNA expression, independent of gD glycosylation status, these data also fit with published observations that binding by HSV-1 is insufficient to cause the up-regulation of interferon-stimulated genes [30]. The up-regulation of IFN-β, albeit it through a different mechanism, is suggestive of a previously unidentified role for gH in eliciting a change in host gene expression. An entry-defective HSV-1 mutant lacking gB that also contains an RGE rather than the integrin binding RGD motif of gH has been constructed and future studies may further elicit the role of gH in interferon stimulation.

The methodology used here required the serum-starvation of primary human fibroblasts for five days. In the absence of serum, primary fibroblasts rapidly enter a quiescent state. As a DNA virus that requires host nuclear factors to replicate its genome, it is therefore not surprising that HSV-1 would stimulate cells from a G0 state into one that would favour DNA replication and possibly promote the transcription of viral genes.

Quiescent cells in vitro have very low levels expression of the transcription factor c-Myc. Its up-regulation is rapidly induced after mitogenic stimulation or the introduction of serum and increased expression of c-Myc is consistent with the advancement of cellular proliferation [41]. Control of myc transcription can be influenced by a number of pathways, including PI3K/Akt signalling, which was shown here to occur as a result of binding by gD. A central role for c-Myc in promoting cell-cycle progression is evident from the genes that it can up-regulate such as E2F2 [42]. Progression of the cell cycle depends on the additional activity of cyclin-dependent kinases. By interacting with the promoters for genes encoding cyclins and cyclin-dependent kinases, c-Myc can influence the advancement on the cell cycle into the G1/S phase [43].

Cyclin-dependent kinase 2 (CDK2) is one such downstream target of c-Myc activity, as well as the Androgen pathway, highlighting the signalling cross-talk that may occur [44]. CDK2 is involved in the progression of the cell cycle from G1 through to S phase. Transient activation of CDK2 was shown to occur early in HSV-2 infection at two hours post-infection, and is crucial in early HSV-1 infection [45][46]. Kinase action by the cyclin A/CDK2 complex liberates the bound transcription factor E2F from Rb, a transcription factor that has previously been shown to be active during HSV-1 infection [47].

Epithelial cells at the initial site of HSV-1 infection in vivo are likely to be in a resting state, necessitating the virus to evolve a pre-entry signalling mechanism by which to stimulate the cell to provide host factors that are necessary for viral replication. We have demonstrated that signalling induced by HSV-1 glycoproteins, primarily gD, has the potential to: activate cellular transcription factors that augment viral gene transcription, differentially express a number of cellular genes so as to condition the cell for optimal replication or, alternatively, signal transduction may occur as a secondary effect to the appropriation of cellular receptors to achieve viral entry.

Supporting Information

Table S1 Normalised datasets for genes differentially expressed on a signalling-specific microarray after infection with entry-defective HSV-1. Found at: doi:10.1371/journal.pone.0009560.s001 (0.14 MB XLS)

Table S2 Primers used in real-time PCR to determine the relative abundance of corresponding mRNA transcripts. Found at: doi:10.1371/journal.pone.0009560.s002 (0.28 MB DOC)

Figure S1 Characteristics of virions treated with PNGase. Found at: doi:10.1371/journal.pone.0009560.s003 (0.76 MB PDF)

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Author Contributions

Conceived and designed the experiments: IJM TM. Performed the experiments: IJM. Analyzed the data: IJM. Contributed reagents/ materials/analysis tools: IJM. Wrote the paper: IJM TM.

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