Inhibition of Breast Cancer Cell Proliferation through Disturbance of the Calcineurin/NFAT Pathway by Human Herpesvirus 6B U54 Tegument Protein

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Nuclear factor of activated T cell (NFAT) proteins are key regulators involved in multiple physiological mechanisms, such as immune response and cell growth. The capacity of selective calcineurin/NFAT inhibitors to decrease NFAT-dependent cancer cell progression, particularly in breast cancer, has already been demonstrated. In this study, we report a role for the human herpesvirus 6B (HHV-6B) U54 tegument protein in inhibiting MCF-7 breast cancer cell proliferation by inhibiting NFAT activation.

The NFAT transcription factors include five elements (NFAT1 to NFAT5) (1, 2), four of which are regulated by Ca^{2+} (NFAT1 to NFAT4) (3, 4). In resting cells, NFAT proteins are hyperphosphorylated and remain cytoplasmic. Upon cell stimulation and increases in intracellular Ca^{2+} concentrations, the Ca^{2+} /calmodulin-dependent serine phosphatase calcineurin (CaN) (5) becomes active and recruits and dephosphorylates NFAT, exposing a nuclear localization signal that allows NFAT to migrate to the nucleus and enhance the expression of several genes (6–9).

Disorders of the CaN/NFAT pathway cause disturbances in adaptive immune responses, cell differentiation, and cell proliferation (10). Selective inhibitors known to prevent CaN/NFAT interaction (11) and subsequent NFAT activation include drugs such as cyclosporine (CsA) (12) and FK506 (tacrolimus) (13), inhibitory peptides (14, 15), and proteins expressed by pathogens, such as the A238L protein of African swine fever virus (16, 17). In the accompanying study, Iampietro et al. identified the HHV-6B U54 tegument protein as being capable of inhibiting NFAT activation and subsequent *IL-2* gene expression, pointing out a role for the U54 protein in immune evasion (18). Considering that the functions of NFAT extend beyond the development of the adaptive immune response, we evaluated the effects of U54 expression in the proliferation of cells whose growth is NFAT dependent.

TABLE 1 Relative expression of NFAT1, -2, and -3 in MCF7 and Jurkat T cells^a

Protein	Expression in ^b :	
	MCF7	Jurkat
NFAT1	+	+++
NFAT2	+	+++
NFAT3	–	++

^a Expression of NFAT transcripts in MCF7 and Jurkat cell lines was evaluated by RT-PCR assay. MCF7 and Jurkat cells were lysed using the Qiazol lysis reagent. RNA was extracted, and RT was performed to obtain cDNAs that were tested with specific primers to evaluate expression of mRNA. Primers used were NFAT1 forward (5'-CGA AGA AGA GCC GAA TGC AC-3') and NFAT1 reverse (5'-AGA AAC TTC TGC GGC CCT AC-3'), NFAT2 forward (5'-CAC TCC TGC TGC CTT ACA CA-3') and NFAT2 reverse (5'-AAG ATG CGA GCA TGC GAC TA-3'), and NFAT3 forward (5'-CGG CCT CTA AGA GAG GTT GA-3') and NFAT3 reverse (5'-CCT CCT TTT CCT CCC CGA AC-3'); primers used for GAPDH were described by Jaworska et al. (32).

^b –, absence of expression; +, basal expression level; ++, moderate expression level; +++, high expression level.

FIG 1 U54 inhibits endogenous NFAT transcriptional activity in MCF-7 cells. Transcriptional activity of endogenous-expressed NFAT factors was evaluated in MCF-7 cells by luciferase assay. MCF-7 cells were transfected with 4TO, 4TO-U54 (Myc), 4TO-U11 (FLAG), 4TO-U54mut (myc), and NFAT-Luc reporter plasmids. As positive control, we pretreated 4TO-transfected cells with CsA. Luciferase activity was determined and normalized to protein content ($n = 4$). Western blot analysis confirmed the expression of proteins of interest for each condition tested. Beta-actin was included as a loading control.

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FIG 2 U54 specifically reduces MCF-7 breast cancer cell growth. MCF-7 cells (A and B) and 293T cells (C) were transfected with 4TO, 4TO-U54 (myc), 4TO-U11 (FLAG), and 4TO-U54mut (myc) plasmids. As controls, we pretreated 4TO-transfected cells with CsA (A and C) or FK506 (B). Cells were harvested and evaluated at 24 h, 48 h, 72 h, and 96 h following transfection by cell counting assay ($n = 4$). Western blot analysis confirmed the expression of proteins of interest for each condition tested. Beta-actin was included as a loading control.

Breast cancer is the leading cause of cancer death in women worldwide (19) and is caused by a disturbance of NFAT activity (20, 21), promoting cell transformation, proliferation, invasion, and tumor angiogenesis (22, 23). Relative expression levels of NFAT members in MCF-7 cells are presented in Table 1. We used

the MCF-7 breast cancer cell line to test the possible inhibitory effects of HHV-6B U54 protein on cell proliferation. To achieve this goal, MCF-7 cells (2×10^5) were transfected with the expression vectors 4TO, 4TO-U54 (encoding wild-type [WT U54]), 4TO-U54mut (IT296-297AA mutant with reduced NFAT inhib-

itory potential), and 4TO-U11 (encoding WT U11) and NFAT-Luc reporter plasmids, as described by Iampietro et al. (18). After 48 h, cells were stimulated with 25 ng/ml TPA (12-*O*-tetradecanoylphorbol-13-acetate) and 0.5 μ M ionomycin (TPA-ionomycin) to induce the CaN/NFAT pathway for an additional 24 h or left unstimulated. Luciferase activity was determined and normalized to protein content ($n = 4$) as described by Iampietro et al. (18). Treatment with TPA-ionomycin activated endogenous NFAT, resulting in a 5-fold increase ($P < 0.0001$) in luciferase activity, while cells expressing U54 showed a 70% reduction in luciferase activity ($P < 0.0001$) (Fig. 1). Cells treated with 5 μ g/ml CsA were used as a positive control. Expression of U54mut or U11, a second HHV-6 tegument protein, had marginal effects on reporter activity. Protein expression was monitored by Western blot analysis with beta-actin as a loading control. Next, we wanted to determine whether the U54 inhibitory activity would translate to a physiological effect, such as reduced cell growth. We transfected 293T and MCF-7 cells (1×10^5) with the plasmids described above and cultured the cells for 96 h. CsA and 5 μ g/ml FK506 were used as positive inhibitory controls. Transfection efficiencies were determined for several wells ($n = 6$) using a green fluorescent protein (GFP) reporter vector and found to be equivalent (data not shown). Cells were counted every 24 h for 4 days using an automatic Cellometer T4 cell counter (Nexcelcom, Lawrence, MA). After 72 h and 96 h, the number of 4TO-transfected MCF-7 cells increased $4.5\times$ and $7.5\times$, respectively ($P < 0.001$) ($n = 4$) (Fig. 2A). Cells transfected with 4TO-U54mut and 4TO-U11 showed proliferation equivalent to that of 4TO control cells. In contrast, at 72 and 96 h posttransfection, MCF-7 proliferation was significantly inhibited by U54 (Fig. 2A). Similar results were obtained with FK-506 (Fig. 2B). 293T cells, which do not rely on NFAT for proliferation (used as controls), were not affected by CsA or U54 expression (Fig. 2C). Protein expression was monitored by Western blot analysis. We next determined how the U54 protein would cause NFAT inactivation, leading to cell growth inhibition. To highlight this mechanism, we analyzed the phosphorylation status of ectopically expressed NFAT1 detected with an antibody detecting the hyperphosphorylated forms (140 kDa) of NFAT1. MCF-7 cells (1.5×10^5) were transfected with 4TO, 4TO-U54, 4TO-U11, 4TO-U54mut, and REP-NFAT1 plasmids. After 48 h, cells were stimulated with TPA-ionomycin for 10 min or left unstimulated. Cells pretreated with 10 μ g/ml CsA were used as a positive control for inhibition of NFAT dephosphorylation. As shown in Fig. 3, under resting conditions, NFAT1 was hyperphosphorylated, as expected. In control-transfected cells (4TO) and in U11- and U54myc-expressing cells, TPA-ionomycin induced the dephosphorylation of NFAT1. In contrast, in the presence of U54 or CsA, NFAT1 remained hyperphosphorylated (Fig. 3A). Densitometric analyses were used to quantify the relative levels of NFAT1 phosphorylation (Fig. 3B).

Lastly, we studied whether U54 would impact the expression of a gene, such as COX-2, whose expression partly depends on NFAT and whose role in cancer progression is well documented (24–26). Under the conditions tested in the experiment whose results are shown in Fig. 3, we evaluated COX-2 mRNA levels following a 24-h stimulation with TPA-ionomycin. A 12- to 14-fold increase in COX-2 mRNA was recorded in 4TO-, U11-, and U54mut-transfected cells ($P < 0.001$) ($n = 4$), while U54-expressing cells and cells treated with CsA showed significantly reduced COX-2 mRNA ($P < 0.0001$) ($n = 4$) (Fig. 4A). Protein expression was

FIG 3 U54 inhibits dephosphorylation of NFAT1 protein. NFAT1 dephosphorylation was evaluated in MCF-7 cells by Western blotting assay. (A) MCF-7 cells were transfected with 4TO, 4TO-U54, 4TO-U11, 4TO-U54mut, and REP-NFAT1 plasmids. As a positive control, 4TO-transfected cells were pretreated with CsA. Western blot analyses were performed by blotting cells under each condition with a phospho-specific anti-NFAT1 antibody (140 kDa) (upper panel). Western blot analysis confirmed expression of U54 (myc), U54mut (myc), and U11 (FLAG) proteins (middle panels). Beta-actin was included as a loading control. (B) Densitometric analysis of P-NFAT1 was performed following Western blot analysis. The P-NFAT1 level in resting cells (4TO) was set at 100%. Following TPA-ionomycin stimulation, P-NFAT1 levels were compared to levels in the respective resting controls (results are from one representative experiment of three independent experiments).

monitored by Western blot analysis. These results confirm the capacity of HHV-6B U54 protein to abrogate NFAT activation and subsequent MCF-7 breast cancer cell proliferation.

In the accompanying work, we identified HHV-6B U54 tegument protein as a new viral protein inhibiting the CaN/NFAT pathway (18), likely favoring immune escape and enhancing viral infection. In this study, we extended these observations by highlighting the capacity of U54 to inhibit breast cancer cell growth. Cancer immunotherapy protocols have demonstrated some effectiveness against virally induced disorders (27), such as in EBV-induced posttransplant lymphoproliferative disorders (28, 29), but the treatment of solid tumors such as breast cancer remains a major challenge. Immunodominant epitopes derived from the HHV-6B U54 protein have been described recently (30, 31). Coupled to our observation that U54 can inhibit cancer cell growth, expression of U54 in breast cancer cells could give a double advantage: first, by limiting cancer cell proliferation, and second, by “marking” the cells for anti-U54 CD8 T cell recognition and destruction. However, before such therapy can be envisioned, sev-

FIG 4 U54 reduces COX-2 gene transcription in MCF-7 cells. (A) MCF-7 cells were transfected with 4TO, 4TO-U54, 4TO-U11, and 4TO-U54mut plasmids for 48 h before being stimulated with TPA-ionomycin for an additional 24 h or left unstimulated. RNA was extracted, and COX-2 mRNA levels were determined by real-time reverse transcription-quantitative PCR (RT-qPCR). The primers used for COX-2 detection were COX-2 forward (5'-TGC ATT CTT TGC CCA GCA CT-3') and COX-2 reverse (5'-AAA GGC GCA GTT TAC GCT GT-3'). The primers for GAPDH, used for normalization, were previously described (32). (B) Western blot analysis confirmed the expression of U54 (myc), U54mut (myc), and U11 (FLAG) proteins. Beta-actin was included as a loading control.

eral more studies are needed to identify a reliable gene therapy approach allowing the specific expression of U54 in breast cancer cells.

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