Interaction of Marek’s disease virus oncoprotein Meq with heat-shock protein 70 in lymphoid tumour cells

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Marek’s disease virus (MDV) is a highly oncogenic alphaherpesvirus that induces the rapid onset of T-cell lymphomas in poultry. The MDV-encoded oncoprotein Meq plays an important role in oncogenicity, as its deletion abolishes the ability of the virus to induce tumours. It has been shown previously that Meq oncogenicity is linked to its interaction with C-terminal binding protein 1 (CtBP), a property also shared by other virus-encoded oncoproteins such as adenovirus E1A and Epstein–Barr virus EBNA3A and -3C. Therefore, this study examined whether Meq also shares the properties of these viral oncoproteins in interacting with other binding partners such as heat-shock protein 70 (Hsp70), a molecular chaperone protein linked to multiple cellular functions including neoplastic transformation. Confocal microscopic analysis demonstrated that MDV infection induced nuclear accumulation of Hsp70 and its co-localization with Meq. Biochemical evidence of Meq–Hsp70 interaction was obtained by two-way immunoprecipitation with Meq- and Hsp70-specific antibodies. To demonstrate further the Meq–Hsp70 interaction in virus-induced lymphomas, recombinant MDV was generated expressing an N-terminal tandem affinity purification (TAP) tag-fused Meq by mutagenesis of the infectious BAC clone of the oncogenic MDV strain RB-1B. Demonstration of Hsp70 in the TAP-tag affinity purified Meq from tumours induced by the recombinant virus, using quadrupole time-of-flight tandem mass spectrometry analysis, further confirmed the Meq–Hsp70 interaction in the transformed lymphocytes. Given the well-documented evidence of the tumorigenic properties of Hsp70 and its interaction with a number of other known viral oncoproteins, demonstration of the interaction of Meq and Hsp70 is significant in MDV oncogenesis.

INTRODUCTION

Current estimates suggest that viruses are involved in 15–20 % of human cancers worldwide (Javier & Butel, 2008). Oncogenic viruses, as efficient inducers of cancers, have helped significantly in understanding the molecular mechanisms of oncogenesis. Marek’s disease (MD), a highly oncogenic viral disease induced by Marek’s disease virus (MDV), is widely seen as a natural model for virus-induced lymphomas (Calnek, 1986; Osterrieder et al., 2006). MDV induces rapid-onset CD4+ T-cell lymphomas within a few weeks of infection, and MD lymphomas have many biological parallels with tumours associated with human herpesviruses such as Epstein–Barr virus (EBV) and Kaposi’s sarcoma herpesvirus (KSHV, human herpesvirus 8). For example, it has recently been shown that MDV and KSHV both encode an oncogenic microRNA-155 orthologue (Gottwein et al., 2007; Zhao et al., 2009), whilst EBV strongly induces host microRNA-155 expression (Yin et al., 2008). However, the most important viral gene associated with MD oncogenicity is Meq (MDV EcoRI Q), encoded from the repeat region (IR1/TR1) of the MDV genome (Jones et al., 1992; Nair & Kung, 2004). The direct role of Meq in MDV oncogenicity has been clearly demonstrated by the abolition of oncogenicity in Meq-deleted mutant viruses (Lee et al., 2008; Lupiani et al., 2004). Although all of the molecular mechanisms of Meq-induced oncogenicity are yet to be identified, an increasing amount of data from various laboratories suggest that the DNA-binding and transcriptional functions of Meq are strongly modulated by its interaction with multiple protein partners (Levy et al., 2005; Nair & Kung, 2004; Osterrieder et al., 2006).

Structurally, the 339 aa Meq protein comprises an N-terminal bZIP domain, a proline-rich unstructured middle region and a C-terminal transactivation domain. The presence of the characteristic bZIP domain allows Meq to form homodimers with itself, as well as to heterodimerize with a number of bZIP proteins, such as c-Jun (Levy et al., 2003). Mutations in the bZIP region that prevent
dimerization abolish viral oncogenicity (Brown et al., 2006), suggesting that partnering with other bZIP proteins is important for Meq oncogenicity. Recent data have also shown that homodimerization alone is insufficient for Meq-induced transformation by MDV (Suchodolski et al., 2009). The N-terminal region of Meq contains a PLDLS motif, also present in other viral oncoproteins such as adenovirus E1A (Chinnadurai, 2002, 2009) and EBV EBNA3A (Hickabottom et al., 2002), with which they interact with the transcriptional co-repressor C-terminal binding protein-1 (CtBP). Meq–CtBP interaction is crucial for MDV oncogenicity, as mutations that abolish the interaction make the virus non-oncogenic (Brown et al., 2006). In addition, Meq is also reported to interact with a number of cellular proteins such as p53 and retinoblastoma (Rb) tumour-suppressor proteins (Brunovskis et al., 1996). Thus, identification of the ‘Meq interactome’ could provide valuable insight into the molecular pathways of Meq function and MDV oncogenicity.

The functions of most proteins, particularly those forming multiprotein complexes, are dependent on their correct folding, a process in which they are assisted by various molecular chaperone proteins. Heat-shock protein 70 (Hsp70) belongs to an important family of molecular chaperone proteins. Heat-shock protein 70 multiprotein complexes, are dependent on their correct function to provide valuable insight into the molecular pathways of Hsp70 in lymphoma cells derived from fresh tumours induced by the virus.

**METHODS**

**Cells and viruses.** Primary chicken embryo fibroblasts (CEFs) prepared from 10-day-old specific-pathogen-free (SPF) embryos (from flocks maintained at the Institute for Animal Health, Compton, UK) were used for the generation and preparation of recombinant virus stocks. MSB-1 is an MDV-transformed CD4+ T-cell line derived from a spleen lymphoma induced by the BC-1 strain of MDV-1 (Akiyama & Kato, 1974). This cell line was grown at 38.5 °C in 5 % CO2 in RPMI 1640 containing 10 % tryptose phosphate broth and 10 % fetal calf serum. Recombinant MDV derived from the self-excisable, US2-restored pRB-1B/X6 virus used in these experiments was generated by transfection of the DNA into CEFs as described previously (Zhao et al., 2008). Other viruses used in the experiment included the wild-type RB-1B virus, RB-1B ΔMeq virus from which both copies of Meq are deleted (Brown et al., 2006) and the recombinant pRB-1B with mCherry protein (kindly received from Dr Roger Tsien, UCSD, California) fused to the N terminus of the pp38 gene (unpublished data).

**Generation of N-terminal Meq TAP tag pRB-1B constructs.** All manipulations for the generation of recombinant viruses were carried out using a modified pRB-1B/X6 bacterial artificial chromosome (BAC) clone (Zhao et al., 2008). A modified TAP tag with double FLAG (FF) and double protein A–IgG binding domains (ZZ) separated by a tobacco etch virus (TEV) protease cleavage recognition sequence in plasmid pIN-X-C-FF-ZZ-A (Tsai & Carstens, 2006) was used to generate recombinant viruses. The FF-ZZ domain was transferred into the pCR8 vector (Invitrogen) to make the Fsp site between the FF and ZZ sequence unique. An I-Sce/Kan cassette was amplified by PCR from pE-P-Kan-S2 (Tscher et al., 2006), using primers Fsp-Sce-Kan-F (5′-CTCTTGCGCATAGGGTAACGCTATCGTTATTATTCAC-3′) and Fsp-Kan-TEV-R (5′-GTGTGGCGCAAGAGCGCGGT-TTGTAGGTACCCCTGAAAAATACAAATTCTGCTACCTGA-CTGTTACACCAATTAAC-3′), with the additional Fsp restriction site and I-Sce recombination sequences. The cassette was inserted into the Fsp site of the pCR8-FF-ZZ plasmid. The FF-(I-Sce/Kan)-ZZ cassette was further amplified by PCR to include the Meq N-terminal homologous sequence and the 3C protease recognition site and the 3C protease recognition site for MDV-1 (Akiyama & Kato, 1974). This cell line was grown at 38.5 °C in 5 % CO2 in RPMI 1640 containing 10 % tryptose...
post-mortem were gently teased apart and the cell suspension purified over Histopaque 1083 (Sigma). Tumour cells positive for both FLAG and Meq were subjected to TAP-tag purification following methods described previously (Tsai & Carstens, 2006). Briefly, about $2 \times 10^7$ tumour lymphocytes were used for nuclear extraction with a NucBuster protein extraction kit (Merck Bioscience). The nuclear extracts were first incubated with anti-FLAG M2 affinity resin (Sigma) and, after three washing steps, the proteins were eluted with $3 \times$ FLAG peptides (Sigma). The eluted protein mixture was further absorbed to protein A beads (GE Healthcare). Glutathione S-transferase–3C protease (purified from bacterially expressed protein) digestion was carried out at $4 °C$ overnight. The resulting protein mixture was resolved on a one-dimensional NuPAGE Novex Bis/Tris gel and stained with SimplyBlue SafeStain (Invitrogen). The protein-containing lanes were excised and divided into 20 equally sized slices. The individual gel pieces were subjected to standard in-gel tryptic digestion (Zimny-Arndt et al., 2009). Briefly, the gel pieces were washed twice in $0.2 \ M \ NH_4HCO_3$/acetonitrile (ACN; 1 : 1) for 30 min at $56 °C$ and, after dehydration, rehydrated with alkylating reagents (55 mM iodoacetamide/0.1 M $NH_4HCO_3$) for 15 min in the dark. The gel pieces were washed twice with $0.1 \ M \ NH_4HCO_3$ and dehydrated again with ACN, followed by vacuum centrifuge drying for 5 min. The dried gel pieces were rehydrated with 0.02 µg trypsin µL$^{-1}$ in $0.1 \ M \ NH_4HCO_3$/10% ACN solution and digested at $37 °C$ overnight. The supernatants were collected and peptides were extracted from the gel pieces with 5% acetic acid/50% ACN. The supernatants and extracts were pooled and dried in a vacuum centrifuge. The peptides were dissolved in 10 µl 0.1% formic acid prior to MS analysis.

**MS analyses.** The tryptic-digested peptides were subjected to capillary high-performance liquid chromatography tandem MS analysis using a 75 µm (internal diameter) × 10 cm BEH 130 C18 column, 1.7 µm particle size (Waters Corp.) and a 60 min gradient: 3–50% solvent B (solvent A: 0.1%, v/v, formic acid in water; solvent B: 0.1%, v/v, formic acid in ACN) on a Waters nanoAcquity UPLC system (final flow rate 400 nl min$^{-1}$) coupled to a Q-ToF Premier tandem mass spectrometer (Waters Corp.). MS analysis was performed in data-dependent acquisition mode and all raw MS data were processed with PLGS version 2.3 ([http://www.waters.com/waters/](http://www.waters.com/waters/)). MASCOT (Matrix Sciences) was used for searching against the chicken protein sequences in the International Protein Index Database and a custom chicken protein database (Ramaroson et al., 2008).

**Immunoprecipitation (IP) and Western blotting.** For the preparation of cell lysates, $2 \times 10^7$ lymphocytes from MD tumours or MSB-1 cells were subjected to nuclear extraction with a NucBuster protein extraction kit (Merck Bioscience). The nuclear extracts were diluted to 1 ml with 50 mM Tris/ HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA and complete protease inhibitor cocktail (Roche). After pre-clearing with 25 µl protein G beads (GE Healthcare), the lysates were incubated at 4 °C for 2 h with a 1:20 dilution of rabbit polyclonal anti-Meq antibody, or 1:500 dilutions of mouse anti-chicken Hsp70 (Stressgen), anti-Myh-9 (myosin) antibody or anti-tubulin antibody (Sigma). After incubating with 25 µl of protein G beads for another 2 h at 4 °C, the beads were washed five times with 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, and the final resin-bound immunoprecipitated proteins were boiled in 2× SDS reducing loading buffer. The precipitated samples were separated on a NuPAGE NoveX BisTris gel, transferred to membrane using an iBlot Dry Blotting System (Invitrogen) and probed with anti-Hsp70 antibody and rabbit anti-mouse horseradish peroxidase (HRP)-labelled secondary antibody. For the detection of Meq, HRP-conjugated anti-Meq mouse monoclonal antibody FD7 (Brown et al., 2006) was used. Signals were detected using ECL Western blotting detection reagents (GE Healthcare). Protein size was determined by running 1 µl of 20–220 kDa protein Magic Markers (Invitrogen).

**Immunofluorescent staining and confocal microscopy.** CEFs seeded onto 13 mm glass coverslips in 24-well plates were infected with 100 p.f.u. wild-type RB-1B or pRB-1B-mCherry-pp38 virus. For heat-shock treatment experiments, CEFs were incubated at 43.5 °C for 2 h and seeded onto coverslips for an additional 16 h of recovery at 38.5 °C. MSB-1 cells in suspension were adhered to glass coverslips using CellTAK Cell and Tissue Adhesive (BD Biosciences). The cells were fixed in 4% paraformaldehyde for 1 h, permeabilized by treatment with 0.1% Triton X-100 for 15 min and blocked with 0.3% BSA in PBS. The cells were stained with a 1:500 dilution of anti-chicken Hsp70 and/or a 1:20 dilution of anti-Meq monoclonal antibody FD7 (Brown et al., 2006) or with anti-pp38 antibody BD1 (Barrow et al., 2003) and detected with Alexa Fluor 488/568-conjugated goat anti-mouse IgG (H + L) antibody (Invitrogen). For double staining, Alexa Fluor 488-conjugated goat anti-mouse IgG1 (γ1) and Alexa Fluor 568-conjugated goat anti-mouse IgG2 (γ2a) were used. Cells were stained with 4',6-diamidino-2-phenylindole (DAPI; 1:20,000) and viewed using a Leica TCS SP5 confocal laser-scanning microscope (Wetzlar) with a 63×/1.4 Plan-Apochromat. Images were scanned at 1024 × 1024 pixels with three-frame averaging and the pinhole adjusted to 1 Airy unit.

**RESULTS**

**MDV infection increases the nuclear accumulation of Hsp70**

Hsp70 is not usually expressed in unstressed cells, and no detectable signals of Hsp70 expression were evident in uninfected CEFs (Fig. 1a). Heat treatment of CEFs at 43.5 °C for 2 h followed by 16 h of recovery resulted in strong Hsp70 induction (Fig. 1b), with the Hsp70 distributed evenly in both the cytoplasm and nucleus. To see whether MDV infection resulted in the specific induction of Hsp70, we examined Hsp70 expression in CEFs infected with the pRB-1B-mCherry-pp38 virus. MDV-infected cells, detected by the mCherry fluorescence, clearly demonstrated a marked increase in the expression of Hsp70 (Fig. 1c). In order to test whether the expression of Meq was responsible for the induction and nuclear accumulation of Hsp70, we examined Hsp70 expression in Meq-deleted virus-infected CEFs. Cells infected with the Meq-deleted virus, identified by pp38 staining using the monoclonal antibody BD1, showed greatly reduced expression of Hsp70 (Fig. 1d). Next, we examined MDV-infected CEFs for evidence of co-localization of Meq and Hsp70. Meq- and Hsp70-specific monoclonal antibodies produced overlapping signals, demonstrating co-localization of the two proteins, particularly in mitotic cells (Fig. 1e). Similar co-localization of the two proteins was also evident in the MDV-transformed lymphoblastoid cell line MSB-1 (Fig. 1f).

**Hsp70 co-immunoprecipitates with Meq in MDV-transformed tumour cells**

Having demonstrated the co-localization of Meq and Hsp70 by confocal microscopy, we sought to obtain
biochemical evidence for the interaction between these two proteins. For biochemical validation of the Meq–Hsp70 interaction in MDV-transformed tumour cells, we carried out IP experiments on MSB-1 cells. First, we asked whether Hsp70 was actually expressed in the nuclear fraction of MSB-1 protein extracts. Western blot analysis of the nuclear extracts prepared from MSB-1 cells showed a single 70 kDa band (Fig. 2a). To test whether native Meq could be co-immunoprecipitated with Hsp70, we used a rabbit polyclonal anti-Meq antibody (Brown et al., 2006) to pull down the Meq protein, and the blot was probed with anti-chicken Hsp70 antibody. This experiment showed clearly detectable signals with Hsp70 (red) and the DAPI-stained nucleus (blue). (d) CEFs infected with RB-1B ΔMeq virus and showing Hsp70 (red) and pp38 (green); the DAPI-stained nucleus is blue. (e) CEFs infected with wild-type RB-1B virus, showing Meq (green), Hsp70 (red) and the nucleus (blue), with the overlay demonstrating co-localization of the two proteins. (f) MDV-transformed MSB-1 lymphoblastoid cell showing Meq (green) and Hsp70 (red) expression, with the overlay showing co-localization. Bars, 25 μm (a); 5 μm (b, f); 7.5 μm (c, e); 10 μm (d).

Recombinant MDV expresses N-terminal TAP tag Meq

In order to examine further whether the Meq–Hsp70 interaction occurs in vivo in MDV-induced tumours in infected birds, we generated a modified virus that expresses an N-terminal TAP-tagged Meq in one of the copies by BAC mutagenesis. We used the modified FF-ZZ tag (Tsai & Carstens, 2006) fused to the N terminus of Meq in the modified RB-1B BAC clone (Zhao et al., 2008), using a two-step Red-mediated recombination method (Tischer et al., 2006). The correct insertion of the TAP tag and the
Expression of the TAP-tagged Meq in infected CEFs was confirmed with Meq-specific (Fig. 3a) and FLAG-specific (Fig. 3b) antibodies. The schematic structure of the TAP–Meq region. Modified virus with N-terminal TAP-tagged Meq gene did not affect its oncogenic properties. To evaluate the expression of TAP-tagged Meq in lymphoma cells, tumour samples from individual birds were tested by Western blot analysis. One of the tumour samples that showed expression of the ~70 kDa TAP-tagged Meq protein detectable with both anti-FLAG and anti-Meq antibodies (Fig. 3c) was selected for TAP purification and further studies.

**Identification of Hsp70 in the affinity-purified Meq complex from tumours**

Nuclear extracts from tumour cells were subjected to two consecutive affinity chromatography steps under native conditions using a modified protocol (Tsai & Carstens, 2006) with 3C protease in the column digest instead of TEV protease. Proteins co-purified with TAP-tagged Meq (Fig. 3f) were separated on an SDS polyacrylamide gel, and each of the protein-containing lanes was excised into 20 equally sized gel pieces and subjected to in-gel digestion with trypsin and MS analysis as described above. Analysis of the MS/MS spectra (Fig. 3g) against the complete International Protein Index Database and a chicken protein sequence database (Ramaroson et al., 2008), identified Hsp70 (NCBI Protein no. NP_001006686) (Fig. 3h) in the TAP affinity-purified sample, demonstrating the interaction of Meq and Hsp70 in the transformed tumour cells.

**DISCUSSION**

The MDV-encoded Meq oncoprotein plays a critical role in the induction of T-cell lymphomas, as Meq-deleted viruses are completely non-oncogenic (Brown et al., 2006; Lupiani et al., 2004). The oncogenic properties of Meq, as with a number of virus-encoded oncoproteins, are closely linked to its interaction with multiple proteins (Nair & Kung, 2004). For example, we have shown previously that Meq interacts with the transcriptional co-repressor CtBP is critical for its oncogenic properties (Brown et al., 2006). CtBP is also an important interaction partner for adenovirus-encoded ElA and the EBV-encoded EBNA3A and -3C proteins, demonstrating the similarities in oncogenic mechanisms of these diverse groups of viral oncoproteins. These observations prompted us to examine whether Meq and other virus-encoded oncoproteins share other binding partners.

The molecular chaperone Hsp70 has been shown to be involved in diverse cellular functions including neoplastic transformation (Dai et al., 2009; Meimaridou et al., 2009). Hsp70 has also been shown to interact with a number of virus-encoded proteins (Forsman et al., 2008; Lum et al., 1992; Young et al., 2008). Here, we demonstrated that Hsp70 is also a binding partner for Meq. The biological consequences of Meq–Hsp70 interaction, and the molecular pathways of Meq function modulated by its interaction with Hsp70, remain to be elucidated. Meq is a bZIP transcription factor; it can homodimerize with itself or heterodimerize with another bZIP transcription factor such as c-Jun or c-Fos. Mutation of the bZIP domain to a homodimer, and Hsp70 interacts with the p53 dimer and influences p53 dimer conformation (Hainaut & Milner, 1997). The transcription factor p53 can also act as a homodimer, and Hsp70 interacts with the p53 dimeric protein by changing the affinity of heterodimers (Carter, 1997). The transcription factor p53 can also act as a homodimer, and Hsp70 interacts with the p53 dimer and influences p53 dimer conformation (Hainaut & Milner, 1992). It is not known whether Hsp70 interaction with Meq can modulate its dimerization properties.
Although the basis for this is not entirely clear, adenovirus E1A has been shown to induce Hsp70 expression in the late S phase and thus is responsible for the cell-cycle control of its expression (Kao et al., 1985). Whilst E1A expression itself is cell-cycle regulated (Kao et al., 1985), there is no direct information available about the relationship between Meq expression and the cell cycle. Nevertheless, Meq is known to associate with a number of cell-cycle regulatory proteins, notably p53, Rb and CDK2, and to promote cell-cycle progression by shortening the G1 phase (Nair & Kung, 2004). On the evidence that p53, Rb and CDK2 are all able to interact with Hsp70 (Cui et al., 2003; Inoue et al., 1995; Lolli et al., 2003), it is likely that the binding of Meq with p53 or CDK2 could be indirect, with Hsp70 bridging the interaction.

One of the distinguishing features of the study described here is the demonstration of the Meq–Hsp70 interaction in T-cell tumours induced by the infectious MDV engineered to express TAP-tagged Meq, using an infection model in the natural target avian host. We believe that this approach using natural infection models of TAP-tagged viruses coupled with MS analysis provides an effective means of discovering protein complexes in the real physiological context, at levels naturally seen in these cells. We are hoping to extend these studies to identify other interacting partners of Meq, with the aim of gaining a deeper understanding of Meq function and its role in oncogenesis. The successful demonstration of Meq–Hsp70 interaction in this study further demonstrates the feasibility of this approach to study global interactions of viral proteins using viruses engineered to express individually TAP-tagged viral genes.

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