Transforming Potential of the Herpesvirus Oncoprotein MEQ: Morphological Transformation, Serum-Independent Growth, and Inhibition of Apoptosis

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Marek's disease virus (MDV) induces the rapid development of overwhelming T-cell lymphomas in chickens. One of its candidate oncogenes, meq (MDV Eco Q) which encodes a bZIP protein, has been biochemically characterized as a transcription factor. Interestingly, MEQ proteins are expressed not only in the nucleoplasm but also in the coiled bodies and the nucleolus. Its novel subcellular localization suggests that MEQ may be involved in other functions beyond its transcriptional potential. In this report we show that MEQ proteins are expressed ubiquitously and abundantly in MDV tumor cell lines. Overexpression of MEQ results in transformation of a rodent fibroblast cell line, Rat-2. The criteria of transformation are based on morphological transfiguration, anchorage-independent growth, and serum-independent growth. Furthermore, MEQ is able to distend the transforming capacity of MEQ-transformed Rat-2 cells through inhibition of apoptosis. Specifically, MEQ can efficiently protect Rat-2 cells from cell death induced by multiple modes including tumor necrosis factor alpha, C2-ceramide, UV irradiation, and serum deprivation. Its antiapoptotic function requires new protein synthesis, as treatment with a protein synthesis inhibitor, cycloheximide, partially reversed MEQ's antiapoptotic effect. Coincidentally, transcriptional induction of bel-2 and suppression of bax are also observed in MEQ-transformed Rat-2 cells. Taken together, our results suggest that MEQ antagonizes apoptosis through regulation of its downstream target genes involved in apoptotic and/or antiapoptotic pathways.
cell lines (6, 42) were maintained as previously described. Dulbecco modified Eagle medium 199-DMEM (1:1) supplemented with 2% chicken serum and 5% calf fibroblasts (CEF) and duck embryo fibroblasts (DEF) were maintained in medium supplemented with 10% calf serum. Chicken embryo fibroblasts (CEF) were maintained undiluted.

Antibodies. Anti-BrdU monoclonal antibody (MAb) (Amersham) was used for 1 h at 37°C followed by fluorescein isothiocyanate (FITC) staining was performed to examine the chromosomal pattern as described by Liu et al. (27).

RESULTS

MEQ proteins are abundantly expressed in MDV cell lines. Although meq transcripts have been detected persistently in the MDV tumor samples and in the cell lines, MEQ’s expression at the protein level has not been addressed due to the lack of avid antibodies. Recently, we generated rabbit antisera against bacterially expressed MEQ (first 168 amino acids) proteins (27). The results of Western blotting are consistent with the expression data based on Northern blots published previously (19). Briefly, MEQ is highly expressed in all MDV tumor proteins (27). The results of Western blotting are consistent with the expression data based on Northern blots published previously (19). Briefly, MEQ is highly expressed in all MDV tumor proteins (27). The results of Western blotting are consistent with the expression data based on Northern blots published previously (19). Briefly, MEQ is highly expressed in all MDV tumor proteins (27).
MEQ is capable of transforming a rodent fibroblast cell line Rat-2 when overexpressed. As described before, several lines of evidence suggest that MEQ may play a role in oncogenesis. First, MEQ’s structure is similar to those of the Jun/Fos oncoproteins and expression of MEQ is heightened in T-cell lymphomas but not in chronically infected cells (19, 47). Second, recent observations have demonstrated that MEQ antisenes transcripts reversed the transformed phenotype of MSB1 (52). However, due to the lack of an efficient chicken T-cell transformation system, it is presently not possible to directly demonstrate the oncogenicity of MEQ in its natural target cells. We therefore utilized Rat-2 fibroblast transformation assays. For this experiment we first introduced meq into the murine retroviral vector pBabe-puro (30) and transfected the resulting construct, pBabe-MEQ, into a packaging cell line, Ψ2. The viral supernatants were then collected and used to infect Rat-2 cells. After selection with puromycin, the positive clones were pooled to avoid clonal variations that might complicate the interpretation of the results. Several passages later, the MEQ-infected Rat-2 cells became morphologically transformed; they are round to deformed and nonrefractile (Fig. 2A). In addition, they were devoid of contact inhibition, and numerous heaped-up foci could be observed. Moreover, they became resistant to trypsinization, presumably due to altered expression of extracellular matrix proteins, and produced huge colonies in soft agar (Fig. 2D). Our indirect immunofluorescence staining (27) and Western blotting (Fig. 1B) showed that MEQ is expressed in these cells at a moderate to high level. Interestingly, three discrete bands were detected by MEQ antisera in pBabe-MEQ-transformed cells as opposed to the broad spliced bands detected in MEQ-infected Rat-2 cells (Fig. 2A). This observation was validated with further experiments using BrdU incorporation (to measure DNA synthesis) and DAPI staining (to analyze chromosomal structure). As shown in Fig. 4C, after serum withdrawal for 3 days, more than 75% of MEQ-transformed Rat-2 cells showed signs of BrdU incorporation and showed mitotic figures as revealed by DAPI staining (Fig. 4B). In contrast, the vector-infected Rat-2 cells underwent either growth arrest (with very little BrdU incorporation) (Fig. 4A’ or apoptosis (Fig. 4A). Apoptosis was confirmed by TdT assay with the ApopTag in situ apoptosis detection kit (Fig. 4C, C’, D and D’). These findings suggest that MEQ-transformed Rat-2 cells either can synthesize their own growth factors through an autocrine loop or may bypass the need of growth factor stimulation through constitutive activation of mitogenic pathways downstream of growth factor receptors. Similar scenarios are often found in the cases for transcription factor-derived oncogenes such as Jun and Fos (2, 11).

MEQ displays antiapoptotic potential. Oncogenic herpesviruses generally encode separate gene products to induce host cell immortalization and/or transformation as well as to block apoptosis (see Discussion). It is therefore possible that MEQ can induce proliferation and show antiapoptotic potential, as it is likely to share similar properties to those of the Jun/Fos family of transcription factors. Indeed, several studies have suggested that MEQ and other MEQ family members may play a role in the regulation of cell proliferation and apoptosis. For instance, a recent study has shown that MEQ can induce cell proliferation in NIH 3T3 cells (55).

MEQ promotes serum-independent growth of MEQ-transformed Rat-2 cells. In addition to the morphological transformation described above, MEQ-transformed Rat-2 cells continued to proliferate in the absence of serum (Fig. 3). This observation was validated with further experiments using BrdU incorporation (to measure DNA synthesis) and DAPI staining (to analyze chromosomal structure). As shown in Fig. 4C, after serum withdrawal for 3 days, more than 75% of MEQ-transformed Rat-2 cells showed signs of BrdU incorporation and showed mitotic figures as revealed by DAPI staining (Fig. 4B). In contrast, the vector-infected Rat-2 cells underwent either growth arrest (with very little BrdU incorporation) (Fig. 4A’ or apoptosis (Fig. 4A). Apoptosis was confirmed by TdT assay with the ApopTag in situ apoptosis detection kit (Fig. 4C, C’, D and D’). These findings suggest that MEQ-transformed Rat-2 cells either can synthesize their own growth factors through an autocrine loop or may bypass the need of growth factor stimulation through constitutive activation of mitogenic pathways downstream of growth factor receptors. Similar scenarios are often found in the cases for transcription factor-derived oncogenes such as Jun and Fos (2, 11).

The above-described experiments implicate MEQ in the abrogation of serum withdrawal-induced apoptosis of Rat-2 cells.

MEQ-mediated BrdU incorporation and inhibition of apoptosis in serum-starved MEQ-transformed Rat-2 cells. BrdU was added to the media of MEQ-transformed and vector-infected Rat-2 cells for 12 h after cells were serum starved for 3 days. Cells were then fixed and stained with anti-BrdU MAb (A’ and B’) and counterstained with DAPI (A and B). Meanwhile, a TdT assay was used to evaluate apoptosis. Briefly, MEQ-transformed and vector-infected Rat-2 cells were also serum starved for 3 days, fixed and stained with ApopTag (C’ and D’) and counterstained with DAPI (C and D). Magnification, ×216.
We then asked whether MEQ is antiapoptotic. Rat-2 cells and their MEQ-transformed counterparts were treated with a number of reagents in addition to being subjected to serum withdrawal. The hallmarks of apoptosis include (i) the formation of distinct ladders of nucleosomal DNA fragments (180 to 200 bp), which can be analyzed by DNA fragmentation or TdT assays, (ii) chromosomal condensation and/or nuclear membrane breakdown, and (iii) formation of apoptotic bodies, which can be evaluated by DAPI staining. The antiapoptotic effects of MEQ were measured as described above.

**Serum withdrawal.** As shown in Fig. 5A and B, in the presence of serum, 0 to 10% of cells are apoptotic in both vector-
infected and MEQ-transformed Rat-2 cells. Upon serum withdrawal for 3 days, 60 to 70% of the vector-infected Rat-2 cells became apoptotic (Fig. 5E), whereas only 5 to 10% of MEQ-transformed Rat-2 cells underwent apoptosis (Fig. 5F).

**TNF-α treatment.** Treatment with mTNF-α (1 ng/ml) under normal serum condition did not induce apoptosis in Rat-2 (vector) cells (Fig. 5C). However, treatment with mTNF-α in the absence of serum rapidly induced apoptosis in vector-infected Rat-2 cells within 18 to 24 h, which represents a significant shortening of the period required for serum withdrawal to induce apoptosis. A total of 60 to 70% of vector-infected Rat-2 cells became apoptotic (Fig. 5G), compared to only 5 to 10% of MEQ-transformed Rat-2 cells (Fig. 5H).

**C2-ceramide treatment.** Similarly, when a downstream mediator of the TNF-α pathway, C2-ceramide (10 μM), was administered in the presence of serum, no apoptosis was induced in vector-infected Rat-2 cells. Meanwhile, the apoptotic effect of serum withdrawal could also be accelerated by C2-ceramide and seemed to be much stronger than that of TNF-α. Apoptosis was observed only 12 h after C2-ceramide treatment in addition to serum withdrawal. Between 70 and 85% of vector-infected Rat-2 cells appeared apoptotic (Fig. 5I), compared to only 0 to 5% of MEQ-transformed Rat-2 cells (Fig. 5J).

**UV irradiation.** Likewise, when cells were subjected to UV irradiation (50 J/m²), 80 to 90% of vector-infected Rat-2 cells underwent apoptosis 24 h later (Fig. 5K); conversely, only 10 to 20% of MEQ-transformed Rat-2 cells were found to be apoptotic (Fig. 5L).

**CHX treatment.** Interestingly, the blocking of TNF-α-induced apoptosis by MEQ apparently requires protein synthesis, as the addition of the protein synthesis inhibitor CHX reduces this block. MEQ did not appear to efficiently block apoptosis induced by CHX (5 μg/ml, 24 h) (Fig. 5N), especially in the presence of TNF-α (Fig. 5P). Under these conditions, there seemed to be more apoptotic cells in MEQ-transformed Rat-2 cells (Fig. 5P) than in vector-infected Rat-2 cells (Fig. 5O). This, however, is due to the fact that most of vector-infected Rat-2 cells treated with CHX and TNF-α underwent apoptosis and were already detached from the petri dish within 12 h.

In summary (Fig. 6), our data strongly suggest that MEQ is capable of antagonizing apoptosis mediated through TNF-α pathways (TNF-α and C2-ceramide treatments), UV irradiation, and serum withdrawal. This antiapoptotic process requires new protein synthesis and is consistent with MEQ being a transcription factor that induces the expression of genes involved in apoptosis, a notion that is supported by experiments described in the following section.

**MEQ up-regulates bcl-2 expression and down-regulates bax expression.** Since MEQ is a transcription factor (39) whose subcellular localization is primarily in the nucleus/nucleolus (27), we postulate that the antiapoptotic effect is likely mediated through regulation of genes involved in apoptosis and/or cell survival at the transcriptional level. Among these, bcl-2 and bax are two candidate genes that are shown to play triggering roles in cell survival and apoptosis, respectively.

As an initial step to explore the possible MEQ-regulated target genes that antagonize apoptosis, we performed Western blotting on vector-infected Rat-2 cells and MEQ-transformed Rat-2 cells to examine the expression levels of Bcl-2 and Bax. As shown in Fig. 7A (lane 3), vector-infected Rat-2 cells express a very low level of Bcl-2 but a high level of Bax. In contrast, MEQ-transformed Rat-2 cells (Fig. 7A, lane 1) expressed significantly higher levels of Bcl-2, while Bax expression was completely turned off. When MEQ-transformed Rat-2 cells were treated with C2-ceramide in the absence of serum, the level of Bcl-2 expression was reduced but was still significantly higher than that of vector-infected Rat-2 cells (Fig. 7A, lane 2). Bax, on the other hand, remains down-regulated (Fig. 7A, lane 2). We further performed an RT-PCR experiment to determine whether the expression of Bcl-2 and Bax is regulated at the transcriptional or translational level. As shown in Fig. 7B, the transcription of bcl-2 is clearly enhanced, whereas the transcription of bax is completely down modulated in MEQ-transformed Rat-2 cells. We were unable to recover enough Rat-2 cells treated with
FIG. 7. Induction of bel-2 and suppression of bax expressions in MEQ-transformed Rat-2 cells. (A) Total cell lysates were prepared from MEQ-transformed Rat-2 cells grown in the presence of serum (lane 1) or after treatment with C2-ceramide in the absence of serum (lane 2) and vector-infected Rat-2 cells (in the presence of serum) (lane 3) and analyzed by SDS-PAGE. The Western blots were stained with rabbit anti-bel-2 and -bax antisera and then detected with a Tropix Western-Light chemiluminescent kit. (B) RT-PCR was performed on total RNA extracted from MEQ-transformed Rat-2 cells grown in the presence of serum (lane 1) or after treatment with C2-ceramide in the absence of serum (lane 2). RT-PCR was similarly carried out with vector-infected Rat-2 cells grown in the presence of serum (lane 3). GAPDH gene expression was used as an internal control for the quality and quantity of the RT-PCR products.

C2-ceramide in the absence of serum to perform either Western blotting or RT-PCR, since most of the cells became apo-C2-ceramide in the absence of serum to perform either Western blotting or RT-PCR, since most of the cells became apo-

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DISCUSSION

In this study we analyzed the transforming potential of MEQ, a putative oncogene of MDV. We show that the meq gene effectively transforms established Rat-2 fibroblasts but only marginally transforms primary embryo fibroblasts (data not shown). Thus, unlike most retroviral oncogenes, but similar to herpesviral oncogenes, MEQ alone is not capable of transforming primary cells and may require additional cooperating oncogenes to display its full transforming activity (10, 14, 21, 28, 48). Nevertheless, our data suggest that MEQ is potentially oncogenic. In our study, injection of chickens with replication-defective virus carrying MEQ yielded a low incidence (5%) of sarcomas, which eventually metastasized to internal organs such as the liver, spleen, and lungs (unpublished results), consistent with its being a weak oncogene.

Oncogenesis is a complex process involving multiple steps, and individual oncogenes act on different steps: some override the G1/S restriction and thus activate cell cycle progression, and individual oncogenes act on different steps: some override the G1/S restriction and thus activate cell cycle progression, and some affect cell-cell communication, and others prevent cell death (4, 23). In this report we show that MEQ is able to induce morphological changes and anchorage-independent growth and to protect transformed cells from apoptosis. MEQ is a transcription factor in the family of the Jun/Fos oncoproteins. MEQ dimerizes with Jun with great affinity and enhances its transactivation (39). We presume that some of the growth stimulation functions may be attributed to MEQ’s ability to activate the oncogene Jun or other bZIP proteins. This hypothesis, however, has yet to be experimentally demonstrated. A striking observation from this study is that Rat-2 cells transformed by MEQ are resistant to apoptosis induced by a variety of stimuli including additions of C2-ceramide or TNF-α, UV irradiation, and serum (growth factor) starvation. Some of these apoptotic regimens utilize common signaling pathways leading to apoptosis. For instance, it has been shown that the sphingolipid ceramide is a downstream effector of TNF receptor (TNF-R) (15). Administration of exogenous C2-ceramide thus effectively activates the apoptotic pathway, by-passing the activation of TNF-R. However, in our study, we found that neither TNF-α nor C2-ceramide alone could induce apoptosis in Rat-2 cells. Efficient induction of apoptosis by these agents occurred only in the absence of serum, which contains growth factors, or in the presence of the protein synthesis inhibitor CHX.

How does MEQ perturb the apoptotic pathway? Analysis of Bcl-2 expression in MEQ-transformed Rat-2 cells reveals significant elevation at both protein and RNA levels. Conversely, the levels of Bax protein and RNA are reduced. It is well documented that overexpression of Bcl-2 in many cell types can antagonize apoptosis induced by serum deprivation, UV irradiation, TNF-α, and C2-ceramide treatments (13, 49). It is thought that Bcl-2 may prevent apoptosis through interaction with the upstream activators (CED-4) of ICE/caspases (9, 44, 51) or by blocking the release of cytochrome c from mitochondria (22, 53). However, the exact mechanisms remain to be elucidated. Bax, on the other hand, is a key effector molecule in executing the apoptotic process (36). In our studies, the induction of bel-2 and the suppression of bax at the transcriptional level appear to correlate with the inhibition of apoptosis in MEQ-transformed Rat-2 cells. If not clear whether this modulation is through direct binding of MEQ to the promoter of these genes or through other factors activated by MEQ. We note that MEQ binding sites (MEREi and -2) are found in the promoter of the human bel-2 gene. Whether the same motifs are present in the promoters of rat bel-2 and bax genes remains to be established. It is possible that other Bcl-2- and Bax-like molecules are regulated by MEQ as well.

While an antiapoptotic property of a viral gene product may impact its transforming potential, most herpesviruses, oncogenic or not, have evolved a number of ways to prevent infected cells from premature cell death during viral replication and/or latency. Several mechanisms have been utilized by herpesviruses to dodge apoptosis of the host cells (for a review, see reference 46). First, some herpesviruses encode Bcl-2 homologs, such as EBV BHRF-1 (17), herpesvirus saimiri ORF16 (43), and human herpesvirus 8 KSbcl-2 (8). These molecules presumably function to antagonize apoptosis in a manner similar to that of Bel-2. Second, some herpesviruses encode p53-binding proteins, such as EBV EBNA-LP (45) and BZLF-1/ZEBRA (54). p53 is known to trigger apoptosis at least in part by transactivating the expression of Bax and down-modulating the expression of Bel-2 (29). Whether these herpesviral p53-binding proteins can inhibit apoptosis through p53 sequestration remains to be elucidated. It is noteworthy that MEQ has been found to interact with p53 in vitro (5). The up-regulation of Bcl-2 and down-regulation of Bax observed in MEQ-transformed Rat-2 cells might thus be mediated by p53 inhibition, although there is very little p53 expressed in Rat-2 and MEQ-transformed Rat-2 cells. Third, some herpesviruses encode death effector domain-containing molecules, such as equine herpesvirus 2 E8 protein, that interfere with signaling transduction of Fas-TNF-R (3, 18) pathways. Last, some herpesviruses encode transcription factors to block apoptosis, such as EBV LMP1 (16, 41), herpes simplex virus type 1 ICP4 (25), and cytomegalovirus IE1 and IE2 (55). LMP1 has been shown to induce the expression of Bel-2 in B cells (16) and A20, a zinc finger protein that can inhibit apoptosis, in epithelial cells (12). The target genes involved in ICP4-, IE1-, and IE2-mediated protection against apoptosis have not been identified yet. Our data add MEQ to the growing list of antiapoptotic herpesviral gene products. While the focus of this report
is on the transforming potential, MEQ may function to prolong the cell life span during MDV replication as well.

In summary, this report provides the first investigation of the biological properties of MEQ, and MDV gene product implicated in oncogenic and latent processes of MDV. MEQ has been well delineated as a transcription factor with the characteristics of nuclear localization, dimerization, transactivation and/or repression, and DNA-binding activity. Here, it is shown that MEQ is mitogenic and antiapoptotic. The former is reflected by its ability to increase growth rate, induce BrdU incorporation, and elicit serum-independent growth. The latter is manifested by its potential to protect cells from apoptosis induced by serum starvation and by treatments with a number of apoptosis-inducing reagents. Our studies provide a framework to understand the mechanisms of MEQ as an effector in MDV oncogenesis.

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