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Adenovirus E3/19K Promotes Evasion of NK Cell Recognition by Intracellular Sequestration of the NKG2D Ligands Major Histocompatibility Complex Class I Chain-Related Proteins A and B**

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The adenovirus (Ad) early transcription unit 3 (E3) encodes multiple immunosubversive functions that are presumed to facilitate the establishment and persistence of infection. Indeed, the capacity of E3/19K to inhibit transport of HLA class I (HLA-I) to the cell surface, thereby preventing peptide presentation to CD8⁺ T cells, has long been recognized as a paradigm for viral immune evasion. However, HLA-I downregulation has the potential to render Ad-infected cells vulnerable to natural killer (NK) cell recognition. Furthermore, expression of the immediate-early Ad gene E1A is associated with efficient induction of ligands for the key NK cell-activating receptor NKG2D. Here we show that while infection with wild-type Ad enhances synthesis of the NKG2D ligands, major histocompatibility complex class I chain-related proteins A and B (MICA and MICB), their expression on the cell surface is actively suppressed. Both MICA and MICB are retained within the endoplasmic reticulum as immature endoglycosidase H-sensitive forms. By analyzing a range of cell lines and viruses carrying mutated versions of the E3 gene region, E3/19K was identified as the gene responsible for this activity. The structural requirements within E3/19K necessary to sequester MICA/B and HLA-I are similar. In functional assays, deletion of E3/19K rendered Ad-infected cells more sensitive to NK cell recognition. We report the first NK evasion function in the Adenoviridae and describe a novel function for E3/19K. Thus, E3/19K has a dual function: inhibition of T-cell recognition and NK cell activation.

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NKG2DLs has been reported to occur in response to stress, including infection, genotoxic stimuli, cell transformation, and heat shock (22, 23). Crucially, HAdV-5 E1A has been demonstrated to upregulate NKG2DLs in both transformed human and rodent cell lines (15, 39), rendering them susceptible to NK cell attack. More recently, we have shown that breakthrough expression from replication-deficient HAdV-5 E1-negative vectors also upregulated NKG2DLs and thereby sensitized vector-transduced cells to NK attack (48). Ad thus encodes E1-dependent and E1-independent functions that act separately to stimulate NKG2DL expression.

The efficient upregulation of NKG2DLs combined with the simultaneous intracellular retention of endogenous MHC-I theoretically should render Ad-infected cells extremely vulnerable to NK cell attack. We therefore sought to investigate the impact of NKG2DL activation during productive Ad infection. Unexpectedly, cell surface expression of MICA and MICB was efficiently suppressed during wild-type (wt) HAdV-5 infection. Ad was shown to encode a function that acts posttranslationalally to sequester both MICA and MICB within the ER. This is the first description of an Ad NK cell evasion function and provides novel insights into the role of E3/19K as a potential virulence factor.

Materials and Methods

Cells. Human fetal foreskin fibroblasts (HFFF), HFFF immortalized with human telomerase reverse transcriptase (37) and transduced with a retrovirus expressing the human coxsackievirus Ad receptor (31) (HFFF-hTERT-hCAR), human embryonic retinoblast 911 cells, and human embryonic kidney 293 cells were all grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Invitrogen). HFFF-hTERT-hCAR were used in all experiments using fibroblasts except those shown in Fig. 1A and are referred to as HF throughout. The 911 MICA-YFP cell line was generated by transfection with a MICA-yellow fluorescent protein (YFP) fusion construct (a kind gift from Daniel Davis, Imperial College, London) (36) and selection with G418 (0.75 mg/ml). The 293 cell lines, E3-45 and E22.7, were established following transfection with the EcoRV C and EcoRI D fragments of HAdV-2, containing all or a part of the E3 region, respectively (30). All E3/19K mutants have been generated in the EcoRI D fragment, which was then transfected into 293 cells together with the neophosphotransferase gene. After selection, 293 transfectants were cultured in Dulbecco modified Eagle medium-10% fetal calf serum containing 200 μg/ml G418. Substituting alanine for the conserved tryptophan in position 96 of HAdV-2 E3/19K created mutant W96A. Transfectants were selected on the basis that they expressed a similar amount of E3/19K as detected by a rabbit antiserum against the cytoplasmic tail of E3/19K (40) or additional monoclonal antibodies (MAbs) (B. Menz and H.-G. Burgert, unpublished). EKE*1 cells express a modified E3/19K molecule with the transmembrane segment replaced by that of MHC-I and the lysines of the ER retrieval signal altered to serines (KKMP3SSMP). This E3/19K mutant is efficiently transported to the cell surface. The cysteine mutants have been described previously (40).
MICA genotyping. Genomic DNA was used to amplify a 2.2-kb fragment of the MICA gene encompassing exons 2, 3, 4, and 5. Sequencing-based typing was performed using primers to sequence exons 2, 3, and 4 in both orientations (29, 55). Sequence analysis and assignment of MICA allele type were performed using AssignSBT (Connexio Genomics, Australia). Ambiguous types were analyzed further if distinguishable by the number of GCT repeats in exon 5 using a reverse sequencing primer to sequence exon 5. The combination of GCT triplets was identified by the heterozygous sequence as described previously (54).

Viruses. The generation of the Ad-Easy-1-based vector RAd592 (48) and the pM17-based vector RAd60 (50) has been described previously. Both of these vectors are E1 deleted, and neither expresses a transgene. Ad-Easy-1-based vectors have a deletion in the E3 region spanning bp 28130 to 30820 of the Ad5 genome, eliminating all E3 open reading frames (ORFs) except 12.5K, which is present but incomplete and thus is likely to be nonfunctional. pM17-based vectors are based on the HAdV-5 dl309 virus, which has a deletion running from bp 30005 to 30750 leading to partial or complete deletion of the 10.4K, 14.5K, and 14.7K ORFs; hence, the protein products of these genes are not expressed (6). wt HAdV-5 was provided by Vivien Mautner, University of Birmingham, United Kingdom. wt Ad5 and the E3/19K mutants were grown and titers were determined on A549 cells. The Ad5-based vectors RAd60 and RAd592 were grown and titers were determined on 293 cells. A549 and 293 exhibit comparable infectivities. Virus-infected cells were recovered by centrifugation and washed with phosphate-buffered saline (PBS), and virus was extracted using Arklane P as described previously (28).

To clone the HAdV-5 genome into a single-copy vector, Ad genomic DNA was isolated from purified virions using the QIAamp blood kit (Qiagen). Purified viral DNA was then cotransformed into SW102 bacteria (49) with a mixture of bacteriophage artificial chromosome (BAC)/pBR322-based vector pBeloBAC11 (New England Biolabs) in which the left (bp 1 to 480) and right (bp 30005 to 35938) ends of the Ad5 genome had been cloned. The BAC construct was linearized with NheI, which cuts between the arms of homology, to promote homologous recombination. Chloramphenicol-resistant colonies were grown, and BAC DNA was extracted and digested with the appropriate enzymes to homologous recombination. Chloramphenicol-resistant colonies were grown and titers were determined on 293 cells. A549 and 293 exhibit comparable infectivities. Virus-infected cells were recovered by centrifugation and washed with phosphate-buffered saline (PBS), and virus was extracted using Arklane P as described previously (28).

RESULTS

Identification of an NK cell evasion function in the HAdV-5 E3 region. The effect of Ad infection on NKG2DL expression was investigated first in primary HFFF. Consistent with previous observations (48), delivery of a replication-deficient HAdV-5 ΔE1,E3 vector clearly induced MICA/B surface expression (Fig. 1A). However, HFFF infected in parallel with wt HAdV-5 did not exhibit an increase in surface MICA/B (Fig. 1A). This finding indicated that HAdV-5 may encode a function to control MICA/B during productive infection. Expression of viral genes from replication-deficient vectors in HFFF is restricted by the E1 deletion. To complement this genetic defect in replication-deficient vectors, the E1-positive 911 helper cell line was infected with two such vectors: RAd592 effectively lacks all E3 functions, whereas RAd60 has a partial deletion (Fig. 1B). 911 cells constitutively express high levels of surface MICA/B, which was efficiently downregulated following infection with both RAd60 and wt HAdV-5 but not with RAd592 (Fig. 1C). Since the function responsible for suppressing MICA/B was retained in RAd60 yet was absent from RAd592, it was provisionally mapped to E3 and more specifically to the 12.5K, 6.7K, 19K, and/or 11.6K ORF (Fig. 1B). 911 cells constitutively express high levels of surface MICA/B, which was efficiently downregulated following infection with both RAd60 and wt HAdV-5 but not with RAd592 (Fig. 1C). Since the function responsible for suppressing MICA/B was retained in RAd60 yet was absent from RAd592, it was provisionally mapped to E3 and more specifically to the 12.5K, 6.7K, 19K, and/or 11.6K ORF (Fig. 1B).

Downregulation of MICA/B is mediated by E3/19K. A panel of 293 cell lines containing the complete HAdV-2 E3 region and targeted deletions therein was exploited to identify the function responsible for suppression of MICA/B surface expression (Fig. 2A). Since HLA-I and Fas are recognized targets of E3/19K and E3/10.4-14.5K (RID), respectively, their expression serves as a measure of E3 function and was determined alongside that of MICA/B. As expected, Fas surface expression was downregulated only in E3-45 cells, as only these cells express an intact RID (10.4K and 14.5K) complex (18). Cell surface MICA/B and HLA-I were efficiently downregulated in both the E3-45 and E22.7 cells; both cell clones express unmodified versions of E3/19K. HAdV-2 E3/19K is a glycoprotein comprising an N-terminal ~105-aa ER-luminal domain, a ~22-aa hydrophobic transmembrane sequence, and a 15-aa cytoplasmic tail (Fig. 2B). E3/19K has two functional entities: the luminal part containing two disulfide bonds essential for binding HLA (40) and a C-terminal ER retrieval signal mediating retrograde transport of E3/19K and associated HLA from the cis-Golgi apparatus to the ER (27). In the EKE*1 cell line, the C-terminal ER retrieval signal has been mutated and the substitutions made in the transmembrane domain so that the modified E3/19K escapes to the cell surface (51). Neither HLA-I nor MICA/B was efficiently downregulated in the EKE*1 cells. Thus, we conclude that E3/19K is responsible for suppressing cell surface expression of MICA/B.
with β₂-microglobulin or presents peptide (23). While MICA and MICB share 84% aa identity, they also exhibit 23 to 35% identity with domains in classical MHC-I heavy chains (32). Within the MHC-I heavy chain, the α1 and α2 domains appear to be critical for E3/19K binding (4, 9, 19, 20). To help identify sequence elements within E3/19K responsible for MICA/B suppression, a series of cysteine mutants of E3/19K were tested. The E3/19K luminal domain contains two intramolecular disulfide bonds between the conserved cysteines Cys11-Cys28 and Cys22-Cys83, which are essential for binding MHC-I, whereas the C-proximal cysteines 101, 109, and 122 are dispensable for this function (40) (Fig. 2B). A similar requirement was observed for control of MICA/B; downregulation was compromised when the four conserved N-proximal cysteines (aa 11, 22, 28, and 83) were replaced by serines, whereas mutation of the three C-proximal cysteines (aa 101, 109, and 122) had no significant effect (Fig. 2D). Some differences in the efficiency of downregulation of HLA and MICA/B were observed (e.g., with the Cys101 mutant). Interestingly, a point mutant with alanine substituted for tryptophan at position 96 (W96A) was unable to efficiently downregulate HLA-I surface expression in 293 cell lines yet substantially suppressed cell surface expression of MICA/B (Fig. 2C).

Characterization of HAdV-5 E3/19K mutants. To evaluate E3/19K function in the context of productive infection, specific mutants were generated. The wt HAdV-5 genome was cloned into a BAC, and the following E3/19K mutants generated using DNA “recombineering”: (i) HAdV-5 ΔE3/19K, incorporating a deletion of aa 2 to 98; (ii) HAdV-5 E3/19KSTOP, with an insertion of a premature termination codon and frameshift after aa 11 in the signal sequence; and (iii) HAdV-5 E3/19KW96A, with an alanine substitution for tryptophan 96 (Fig. 3A). Ablation of E3/19K expression in cells infected with HAdV-5 ΔE3/19K and HAdV-5 E3/19KSTOP was confirmed by Western blotting (Fig. 3B). The point mutation in the E2-W96A cell line, associated with downregulation of MICA/B but not HLA-I (Fig. 2C), was essentially replicated in HAdV-5 E3/19KW96A; this modified protein was expressed in similar abundance by the mutant virus (Fig. 3B).

MICA/B surface expression was profoundly upregulated in HF (telomerase-immortalized human fibroblasts expressing hCAR) infected with the HAdV-5 mutants ΔE3/19K and E3/19KSTOP relative to the wt parental virus or mock-infected cells (Fig. 3C). Further analyses, using antibodies that could differentiate between MICA and MICB in flow cytometry, demonstrated comparable upregulation of MICA and MICB following infection with both HAdV-5 E3/19K-negative viruses that was not apparent with the parental wt virus. E3/19K-specific downregulation of both MICA and MICB was also observed in the continuous cell lines detailed in Fig. 2A when resolving antibodies were used to monitor surface expression (data not shown). The HAdV-5 E3/19KW96A mutant effectively reproduced the findings obtained with the E2-W96A cell line in that there was a substantial downregulation of MICA and MICB surface expression whereas the prevention of HLA-I maturation was less effective (see Fig. 4C). Most significantly, productive Ad infection was demonstrated to stimulate the efficient induction of NKG2DLs, with MICA and MICB surface expression being prevented only by the action of E3/19K.

E3/19K promotes evasion of NK recognition. The generation of HAdV-5 E3/19K mutants enabled the role of E3/19K in modulating NK cell recognition to be evaluated in a functional assay. The CD107a mobilization assay detects lysosome-associated membrane protein-1 released to the surface of NK cells following degranulation and therefore provides a direct readout of NK cell function. IFN-α-activated PBMC were incubated (6 h) with HF infected with HAdV-5 and the various E3/19K mutants (48 h p.i.), and NK cells (defined as CD3-CD56+CD16+) were assessed for CD107a expression by flow cytometry. A representative result from three independent experiments performed is depicted in Fig. 3D. Differences in CD107a expression on NK cells following incubation with mock- and Ad-infected cells were analyzed using the analysis of variance test with Bonferroni’s posttest. This indicated that the ∼2-fold increase in CD107a expression following incubation with the HAdV-5 ΔE3/19K and E3/19KW96A mutants compared to wt infection was statistically significant (P < 0.05 and P < 0.01, respectively). The difference in CD107a expression following incubation with cells infected with wt virus or the W96A E3/19K mutant was consistent but not statistically significant and thus should not be given weight. Since MHC-I is known to be a ligand for multiple NK cell inhibitory receptors, its downregulation by E3/19K may be expected to stimulate NK recognition. However, only a modest activation was observed with the wt HAdV-5 relative to mock-infected controls. In contrast, functional inactivation of E3/19K by deletion or mutation resulted in a marked stimulation of NK cell activation. Thus, protection against NK cell recognition in the context of a productive HAdV-5 infection is mediated by E3/19K expression.
E3/19K promotes MICA/B retention in the ER. To investigate the effect of E3/19K on posttranslational processing of MICA/B, extracts were prepared from the panel of E3-expressing cell lines (detailed in Fig. 2A) and subjected to EndoH or PNGase F treatment (Fig. 4A). EndoH will cleave only molecules containing high-mannose sugars, whereas those having acquired complex-type carbohydrates during the transport through the medium/trans-Golgi apparatus become resistant. Thus, EndoH resistance is indicative of MICA/B transport through the medium/trans-Golgi apparatus. As a control, extracts were also treated with PNGase F, which cleaves all N-linked carbohydrates. In the parental 293 cell line, MICA/B was detected as a diffuse EndoH-resistant, 60-kDa protein, with two species (40 and 33 kDa) observed following PNGase F digestion. 293 cells are homozygous for the truncated MICA*008 allele that lacks a cytosolic domain. The apparent molecular mass of the faster-migrating species (33 kDa) detected in Fig. 4A is consistent with it being the deglycosylated form of MICA*008, predicted to be 35 kDa. In contrast, in cells expressing wt E3/19K (E3-45 and E22.7) a lower-molecular-mass EndoH-sensitive species of ~48 kDa was apparent, indicating incomplete posttranslational processing of MICA/B. MICA/B was resistant to EndoH digestion in cells expressing the E3/19K mutants EKE*1 and C83S; both forms of E3/19K were also impaired in suppressing MICA/B cell surface expression (Fig. 2C). In the W96A cell line, both the EndoH-sensitive and EndoH-resistant forms were present, with the EndoH-sensitive form predominating. This is consistent with this point mutant possessing an intermediate phenotype.

To corroborate these results during virus infection, Western blot experiments were also performed on extracts prepared from virus-infected cells. HAdV-5 infection resulted in enhanced expression of an EndoH-sensitive form of MICA/B (Fig. 4B) and HLA-I (Fig. 4C); HF consistently exhibit extremely low levels of constitutive MICA/B expression, which is clearly upregulated upon Ad infection. Thus, mature EndoH-sensitive MICA/B forms of ~70 kDa were more readily detected following infection with the HAdV-5 E3/19K mutants.
(ΔE3/19K and E3/19KSTOP) than in the uninfected controls or in wt infection. The E3/19KW96A mutant was again associated with an intermediate effect on MICA/B (Fig. 4B). Samples were immunoblotted for actin as a loading control (Fig. 4D). When expressed in continuous 293 cell lines or in the context of productive Ad infection, E3/19K of species C Ads (HAdV-2 and HAdV-5) acts to inhibit posttranslational maturation of MICA and MICB.

The differential migration of deglycosylated MICA/B in the two cell types can be attributed to allelic variation. While 293 cells are homozygous for the MICA*008 allele, HF carry full-length alleles (MICA*016,*027). Further studies with the
HAdV-5 E3/19K mutants in additional genotyped cell lines (positive for MICA*004, MICA*018, and MICA*009/01/*049) have demonstrated that E3/19K is capable of acting on a broad range of MICA alleles (data not shown).

Colocalization of E3/19K and MICA. To monitor the intracellular localization of MICA during the course of an HAdV-5 infection, a plasmid encoding a MICA-YFP fusion protein was transfected into 911 cells, where cell surface expression was readily detected and confirmed by flow cytometry (not shown). Following infection with HAdV-5, E3/19K expression was predominantly associated with the cell surface (Fig. 5, top two rows). In contrast, in cells infected with wt HAdV-5 virus, the MICA-YFP fusion protein was found in distinct intracellular foci that overlapped significantly with E3/19K staining (Fig. 5, bottom two rows). These foci were found within calnexin-labeled areas of the cell, consistent with the E3/19K:MICA complex being retained within the ER. Indeed, calnexin appears to be enriched in these foci. These foci do not colocalize with any other intracellular compartment tested, including endosomes, the Golgi apparatus, and caveolae (not shown). The intracellular distribution of MICA-YFP following HAdV-5 infection is consistent with E3/19K sequestration of MICA/B in the ER.

**DISCUSSION**

E3/19K was shown to inhibit terminal glycosylation and to downregulate cell surface expression of MHC-I more than 20 years ago (8). In addition to conferring protection against MHC-I restricted cytotoxic T cells, we now show that the same Ad gene product protects against NK cell attack by intracellular sequestration of the NK cell activating ligands MICA and MICB. MICA/B downregulation from the cell surface was demonstrated in continuous cell lines expressing HAdV-2 E3/19K protein and with HAdV-5 E3/19K in the context of lytic virus infection. All characterized human Ad serotypes in species B, C, D, and E encode an E3/19K homolog (7). Suppression of both HLA-I and MICA/B requires both an ER retention/retrieval signal and formation of critical intramolecular disulfide bonds; these features are conserved in E3/19K across the different human Ad subtypes (11). Additional studies have shown this NK cell evasion function is intact in HAdV-4 (species E) (data not shown).

Although E3/19K and MICA/B colocalize, it has not yet been demonstrated that the proteins interact directly. Hence, the possibility that the interaction between E3/19K and MIC is mediated by a cellular intermediate cannot formally be ruled out. However, though qualitative phenotypic differences were observed, virtually all cysteine E3/19K mutants exhibited a simultaneous modulation of HLA-I, MICA, and MICB. The E3/19K point mutant (W96A) provided some discrimination by inducing suppression of MICA/B yet minimal downregulation of HLA-I in continuous cell lines. Potentially, the tryptophan at position 96 may preferentially promote recognition of HLA-I. This disparity was also observed, although it was less pronounced, when the equivalent substitution was introduced into the HAdV-5 genome. Further E3/19K mutagenesis will show whether other amino acids are preferentially required for HLA and MICA/B downregulation. In view of the limited amino acid identity in the extracellular domains between MIC and HLA alleles (~25%), it seems likely that E3/19K is recognizing a conserved structure in these molecules. In this context, it is remarkable that E3/19K can accommodate the extreme polymorphism associated with HLA-I, MICA, and MICB.

MICA and MICB are cell surface glycoproteins with MHC-I-like α1, α2, and α3 domains, yet neither associates with β2-microglobulin or presents peptide (23). We have previously shown that E3/19K can bind MHC-I heavy chains in the absence of β2-microglobulin and prior to folding of the α1 and α2 domains (34, 41). E3/19K recognition of HLA-I also proceeds independently of peptide binding in vitro (34). Thus, E3/19K appears to bind at an early stage during the assembly of MHC-I-like proteins, presumably at the level of calnexin interaction. In this study, we used calnexin as an ER marker together with a MICA-YFP fusion protein to help identify the intracellular site of MICA retention. The MICA-YFP and E3/19K were observed to colocalize with calnexin in bright foci within the cells. In most cell types E3/19K exhibits a reticular ER-like staining rather than a focal distribution, and the concentration of complexes into foci may be a consequence of MICA-YFP overexpression. Nevertheless, the association of calnexin with these foci is consistent with ER retention.

Human cytomegalovirus (HCMV) encodes a number of functions that modulate NK cell recognition (16, 46, 47), including at least three genes that specifically target NKG2DLs. UL142 downregulates MICA alleles but not the truncated MICA*008 allele, and it does not affect MICB (13). UL16 encodes an ER-localized glycoprotein that acts to promote intracellular retention of MICB, ULBP1, and ULBP2 (16). In
addition, a recent report indicates that an HCMV-encoded microRNA specifically targets the 3’ untranslated region of the MICB transcript (43). Although E3/19K and HCMV gpUL16 both promote ER retention and accumulation of NKG2DLs, their functions are quite distinct. E3/19K targets both MICA and MICB with similar efficiency and does not affect ULBP1 to -3 (data not shown), whereas UL16 does not target MICA or HLA-I. E3/19K thus has a novel function in being able to target a broad range of MICA and MICB alleles; in particular, it can target the MICA*008 allele, which is the most commonly expressed allele in populations studied to date (35, 38). It has been speculated that the MICA*008 allele may have emerged as a result of the host response to viral evasion functions acting on MICA (53). However, E3/19K can clearly retain both full-length and truncated MICA alleles and thus represents the first description of a viral protein that can downregulate surface expression of the commonly expressed MICA*008 allele.

The identification of this novel function for E3/19K has implications for the design and application of Ad vector systems (replication deficient and replication competent) and therapeutic oncolytic viruses. Murine models are used extensively to evaluate Ad vectors and oncolytic viruses in vivo. While a comparable murine NKG2D receptor exists, the NKG2DLs have diverged so there is no direct equivalent of MICA and MICB. It should therefore be borne in mind that the E3/19K-mediated NK cell evasion function described here would not be expected to operate in mice. NKG2D also acts as a costimulatory molecule for CD8 T cells (3), and E3/19K expression has been shown to protect infected cells from cytotoxic T-lymphocyte attack (1, 10, 21). Previously this was attributed solely to its ability to downregulate surface MHC-I, but the intracellular retention of potential costimulatory ligands by E3/19K can be expected to contribute to this effect. NKG2D is expressed on additional important immune effector cells, including γδ T cells and IFN-producing killer dendritic cells (3, 14). Thus, the inhibition of NKG2D surface expression by E3/19K is expected to affect the recognition of Ad-infected cells by multiple effector cells of both the innate and adaptive immune systems.

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