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A novel class of herpesvirus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition

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Kaposi's sarcoma-associated herpesvirus encodes two transmembrane proteins (modulator of immune recognition [MIR1 and MIR2]) that downregulate cell surface molecules (MHC-I, B7.2, and ICAM-1) involved in the immune recognition of infected cells. This downregulation results from enhanced endocytosis and subsequent endolysosomal degradation of the target proteins. Here, we show that expression of MIR1 and MIR2 leads to ubiquitination of the cytosolic tail of their target proteins and that ubiquitination is essential for their removal from the cell surface.

MIR1 and MIR2 both contain cytosolic zinc fingers of the PHD subfamily, and these structures are required for this activity. In vitro, addition of a MIR2-glutathione S-transferase (GST) fusion protein to purified E1 and E2 enzymes leads to transfer of ubiquitin (Ub) to GST-containing targets in an ATP- and E2-dependent fashion; this reaction is abolished by mutation of the Zn-coordinating residues of the PHD domain. Thus, MIR2 defines a novel class of membrane-bound E3 Ub ligases that modulates the trafficking of host cell membrane proteins.

Introduction

Viruses of the herpesvirus family are important human pathogens that produce a wide variety of disease states linked to persistent infection of the host. Although many factors underlie viral persistence, one important contributor is thought to be the elaboration of virally encoded molecules that promote evasion of host cell–mediated immunity (Ploegh, 1998). Elegant studies of cytomegalovirus (Wiertz et al., 1997; Hengel et al., 1998), HSV-1 (York et al., 1994), and MHV-68 (Hengel et al., 1999) infection have revealed that such proteins typically target host MHC class I (MHC-I) polypeptides and lead to downregulation of their surface display, thereby impairing recognition of infected cells by host cytotoxic T lymphocytes. The several viral proteins involved in this activity employ different mechanisms to accomplish this task. Some impair the function of the peptide transporter (TAP) that allows the charging of assembling MHC-I chains with antigenic peptides (Fruh et al., 1995), whereas others lead to retention of newly made MHC-I chains in the ER (Ahn et al., 1996; Jones et al., 1996), their relocation to the cytosol for proteosomal degradation (Wiertz et al., 1996), or their trafficking to the lysosome during export (Reusch et al., 1999).

Kaposi's sarcoma-associated herpesvirus (KSHV)* (also called human herpesvirus 8) is a lymphotropic herpesvirus that is strongly implicated in the pathogenesis of Kaposi's sarcoma and two AIDS-related lymphoproliferative syndromes (Whitby and Boshoff, 1998). We (Coscoy and Ganem, 2000, 2001) and others (Ishido et al., 2000a,b) have shown recently that the viral genome contains two genes, K3 and K5, that encode transmembrane proteins (now named modulator of immune recognition [MIR1 and MIR2]) that are involved in the downregulation of MHC-I and other molecules (B7.2 and ICAM-1) involved in immune recognition (see below). The pathway of this downregulation is novel: the target proteins are synthesized efficiently and assembled in the endoplasmic reticulum, acquire their N-linked glycans with normal kinetics, and reach the cell surface normally. However, from that locale they are endocytosed rapidly and efficiently, as judged both morphologically and by the fact that blockade of endocytosis with dominant negative mutants of dynamin restores surface expression of the chains.

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*Abbreviations used in this paper: GS, glutathione-Sepharose; GST, glutathione S-transferase; KSHV, Kaposi’s sarcoma-associated herpesvirus; MIR, modulator of immune recognition; TM, transmembrane; Ub, ubiquitin; wt, wild-type.
Once internalized, the chains are subsequently degraded in the endolysosomal compartment, a reaction that is blocked by chloroquine, ammonium chloride, and other inhibitors of endolysosomal acidification (Coscoy and Ganem, 2000).

MIR1 and MIR2 are type III transmembrane proteins whose NH\textsubscript{2}- and COOH-terminal regions project into the cytosol (unpublished data). The NH\textsubscript{2}-terminal region of both proteins harbors a Zn finger of the PHD/LAP subfamily (Nicholas et al., 1997). This domain is essential for MHC-I downregulation, as is a second cytosolic element located in the COOH-terminal domain (unpublished data). Although the overall pathway in which they function is now clear, MIR1 and MIR2 have slightly different target specificities (see below), and the molecular mechanisms by which they act have therefore been obscure. Here, we show that an important target for MIR2-mediated downregulation resides in the cytosolic tail of the B7.2 molecule. By mapping the target residues, we uncovered that lysine residues in the tail are critical for MIR2-mediated regulation and that this process is tightly linked to ubiquitination of these residues. In vitro experiments indicate that the NH\textsubscript{2}-terminal region of MIR2 can direct the ubiquitination of a substrate protein in the presence of purified functional E1 and E2 enzymes. These results indicate that MIR2 is the prototype of a new family of membrane-bound E3 Ub ligases whose activity influences the trafficking of host cell membrane proteins.

**Results**

**Mapping the determinants of MIR action on its B7 target**

To better understand the mechanisms of MIR-mediated regulation, we mapped the determinants on the target host protein that are required for this regulation. For this purpose, we took advantage of the fact that MIR2 can downregulate the cell surface protein B7.2 (Ishido et al., 2000a; Coscoy and Ganem, 2001) (a costimulatory molecule important in T cell activation) but not its closely related homologue B7.1 (Fig. 1 A). (Interestingly, neither protein can be efficiently downregulated by MIR1 [Fig. 1 A; Ishido et al., 2000a; Coscoy and Ganem, 2001].) Accordingly, we constructed chimeras between B7.1 and B7.2 and tested their ability to be downregulated by MIR2 expression (Fig. 1 B). HeLa cells were stably transduced with each of the B7.1/B7.2 chimeras diagrammed in Fig. 1 B. These chimeras were
found to be stable and were expressed at normal levels on the cell surface in the absence of MIR2 (unpublished data). Next, HeLa cell expressing each of these chimeras were stably transduced a second time with either an empty vector (pMX-pie) or an expression vector for MIR1 or MIR2 (here expressed as functional GFP fusion proteins with plasmids pB-MIR1/EGFP and pB-MIR2/EGFP, respectively) and levels of the chimera on the cell surface measured by flow cytometry. As summarized in Fig. 1 B, the ability of the chimeras to be downregulated by MIR2 mapped uniquely to the cytosolic tail of B7.2, which was both necessary and sufficient to confer downregulation. Interestingly, several of the chimeras acquired the ability to be downregulated by MIR1, a fact to which we shall return in the Discussion.

For higher resolution mapping of the target sequences, we introduced deletions into the cytosolic tail of B7.2 and examined their effects on MIR2-mediated downregulation. Fig. 2 A depicts the deletions employed; as shown in Fig. 2 B, all deletion mutants were stably expressed on the cell surface at normal levels in the absence of MIR2 (pMX-pie control). In the presence of MIR2, mutant B7.2/H9004-1 was strongly downregulated, but mutant B7.2/H9004-2 was completely unaffected. Examination of the amino acid sequence of the two mutants revealed the presence of a lysine-rich segment that is present in B7.2/H9004-1 but absent in B7.2/H9004-2. Interestingly, mutation of these four contiguous lysine residues to alanine in the context of the intact cytosolic tail (mutant B7.2-7A) did not abolish MIR2-mediated endocytosis (Fig. 2 B). This indicates that there are likely to be multiple redundant signals in the cytosolic tail; in this connection, we note that B7.2-7A retains seven additional lysines in its intracytoplasmic domain.

To better define the contribution of lysine residues to MIR2-mediated endocytosis, we examined the regulation of the class I MHC allele HLA-B7 whose cytosolic tail harbors only two lysines (at positions 332 and 337). Each of these was singly mutated to arginine (to minimize changes in charge or basicity); in addition, we constructed the corresponding double mutant. Each construct was first transduced into BJAB cells; the resulting neomycin-resistant cell population was then transduced a second time with either pMX-pie (vector control) or pB-MIR2/EGFP, a vector expressing a functional MIR2-GFP fusion protein. 2 d later, cells were examined by flow cytometry for GFP and surface MHC-I expression. As shown in Fig. 3, cells expressing MIR2-GFP strongly downregulated surface expression of wild-type (wt) MHC-I and each of the single lys/arg mutants; however, the double mutant was completely refractory to MIR2 regulation.

MIR2 expression results in ubiquitination of its targets

In contemplating what functions of lysine might not be supported by an identically positioned arginine residue, it occurred to us that post-translational modification by Ub (Pickart, 2001) (or by Ub-like modifiers [Melchior, 2000]) might behave in this fashion. Although ubiquitination is most widely recognized as a strategy for targeting proteins for degradation in cytosolic proteosomes, recent studies indicate that this modification can serve other functions in both yeast and mammalian cells (Hicke, 2001b). Particularly relevant are recent reports that certain mammalian cell
surface receptors that are known to undergo ligand-mediated downregulation (e.g., those for EGF and human growth hormone) are ubiquitinated on their cytosolic tails during this process, and strong evidence indicates that this modification is required for receptor downregulation (Lee et al., 1999; de Melker et al., 2001). In many of these cases, the cytosolic RING finger-containing protein cbl has been implicated as the likely E3 Ub ligase that catalyzes the transfer of Ub to the receptor (Waterman et al., 1999).

Accordingly, we looked for evidence that B7.2 and MHC-I chains might undergo ubiquitination in the presence of MIR proteins. HeLa cells stably expressing B7.2 were transduced with pMX-pie, pB-MIR1/EGFP, or pB-MIR2/EGFP. After puromycin selection, chains were immune precipitated with anti-B7.2 antibody, and the resulting precipitates were fractionated by SDS-PAGE, transferred to membranes, and blotted with anti-Ub antibody. As shown in Fig. 4 (right), no ubiquitinated forms were observed in vector or MIR-1-expressing cells. However, in MIR2-expressing cells, ubiquitination of B7.2 chains was readily detectable, producing a characteristic heterogeneous array that is presumably due to either polyubiquitin addition, ubiquitination of multiple target lysines, or both. Similarly (Fig. 4, left), cells expressing either MIR1 or MIR2 display ubiquitination of their endogenous MHC-I chains, whereas such chains are not detected in control cells lacking expression of either MIR1 or MIR2.

These results demonstrate a striking correlation between the presence of cytosolic lysines, ubiquitination of the cytosolic tail, and the ability to be endocytosed. They reveal that such lysines are necessary for MIR-induced endocytosis but do not establish that they are sufficient for this activity. To examine this question, we created by mutation a stretch of three contiguous lysine residues in the cytoplasmic tail of B7.1, which normally has no lysines in its intracytoplasmic region.

Figure 3. The presence of lysines in the MHC-I intracytoplasmic region is required for MIR2-mediated downregulation. Lysine 332 and/or lysine 337 within the HLA.B7 cytoplasmic region were mutated to arginine, and the resulting constructs was stably expressed in BJAB cells. Cells were then transiently transduced a second time with either the control vector pMX-pie or pB-MIR2/EGFP, a vector expressing a functional MIR2–GFP fusion protein. Cell surface expression of HLA.B7 wt or mutant molecules was analyzed by flow cytometry.

Figure 4. MIR1 and MIR2 mediate ubiquitination of their targets. wt HeLa cells or HeLa cells expressing B7.2 were stably transduced by pMX-pie, pB-P-MIR1/EGFP, or pB-P-MIR2/EGFP. B7.2 molecules (right) or MHC-I molecules (left) were immunoprecipitated and their ubiquitination status was determined by Western blot analysis using an anti-Ub antibody.

Figure 5. Addition of lysine into B7.1 enable its downregulation. (A) Sequence of the TM and intracytoplasmic regions of B7.1 (top line) and B7.1KKK mutant (bottom line). The mutant residues within the cytoplasmic region of B7.1KKK are indicated in boldface. (B) B7.1 wt and KKK mutant constructs were stably expressed in HeLa cells. Cells were transduced a second time by pB-P-MIR2/EGFP, and surface expression of the B7.1 molecules was analyzed by flow cytometry. (C) Cells expressing B7.1 wt or KKK mutant proteins were stably transduced by pMX-pie, pB-P-MIR1/EGFP, or pB-P-MIR2/EGFP. From each set of transductants, B7.1 molecules were immunoprecipitated and analyzed by Western blot using an anti-Ub antibody.
domain and is not endocytosed in response to MIR2 expression (Fig. 5 A). When expressed in cells bearing functional MIR2, the wt molecule is present at readily detectable levels on the cell surface, but the mutant displays clear downregulation (Fig. 5 B). As expected, when the B7.1 KKK mutant was immunoprecipitated with anti-B7.1 and blotted with anti-Ub, ubiquitinated forms of the protein were readily detected, whereas no ubiquitination of wt B7.1 was observed (Fig. 5 C).

How does MIR protein expression lead to ubiquitination of its target MHC or B7 molecules? Ubiquitination is a modification performed by a cascade of cytosolic enzymes. So-called E1 (Ub-activating) enzymes activate Ub in an ATP-dependent reaction that couples Ub to the E1 active enzyme (Fig. 5 C). The final step is the transfer of the Ub to the E2 (Ub-conjugating) enzyme. The Ub moiety is then transferred to a second protein, the E2 (Ub-conjugating) enzyme. The Ub group of the substrate lysine. This reaction rarely proceeds directly; typically, it requires catalysis by a third class of enzymes, the E3 Ub ligases. These proteins bind both the E2 and the target, promoting a complex that brings the E2 and the substrate into proximity. Interestingly, MIR proteins contain Zn fingers of the PHD family, structural motifs thought to be involved in protein–protein interactions (Linder et al., 2000; Pascual et al., 2000; Yochum and Ayer, 2001). Our previous work has shown that these variant Zn fingers are located in the NH2-terminal cytoplasmic domain of the protein (Coscoy and Ganem, 2000) and so would be available for E2 interactions. Moreover, mutations that disrupt the PHD/Zn finger domain ablate MIR-induced MHC downregulation (Ishido et al., 2000a; unpublished data). So the simplest model is that MIRs might serve as E3 enzymes that direct the E2 Ub-conjugating enzymes to MHC-I and B7-2, much as cbl directs E2s to the cytosolic tails of activated growth factor receptors. This is a particularly attractive model, since the PHD family of Zn fingers are very closely related structurally to the RING fingers (Capili et al., 2001) found in many cytosolic E3s (e.g., cbl).

Accordingly, we tested the ability of wt and PHD mutant forms of MIR2 to functionally interact with a series of known eukaryotic E2 Ub-conjugating enzymes. In one standard assay of such interaction, in vitro incubation of glutathione S-transferase (GST)–E3 fusions bearing the RING finger region with active E1 and E2 enzymes leads to transfer of Ub to the GST region of the fusion protein (Joazeiro et al., 1999). To see if MIR2 could function in such an assay, we fused the NH2-terminal cytosolic region of MIR2 (bearing its PHD domain) to GST and expressed the resulting fusion protein in Escherichia coli. In parallel, a MIR2–GST fusion bearing mutations in key cysteine residues of the PHD/Zn finger was expressed. Both proteins were partially purified by binding to glutathione-Sepharose (GS), and equal amounts of each was added to a reaction mix containing ATP, Ub, purified E1, and the human E2 UbcH5a. After incubation, the resulting GST-containing protein products were purified by GS binding, separated by SDS-PAGE, transferred to filters, and probed with anti-Ub antibody. As shown in Fig. 6, in the presence of the wt MIR2–GST fusion a prominent band of high molecular weight products reactive with anti-Ub was observed; these products were not detected in reactions programmed with GST alone or with the MIR2–GST fusion protein bearing point mutations in the PHD domain. As expected, these ubiquitinated reaction products were dependent on the presence of ATP and E2 enzyme (Fig. 6).

The transmembrane (TM) region of the target protein plays a role in the MIR2-mediated target selection

The results presented thus far have focused upon the role of the cytosolic tail of the target protein in MIR-mediated regulation, and this role that can now be understood in terms of the provision of substrate lysines for ubiquitination by E2s recruited by MIR2’s PHD domain. However, interaction with E2s is only one function of an E3. Another key role an E3 must play is target recognition, and in most E3s this function is subsumed by a different domain than that involved in E2 recruitment. We have found recently that the TM domains of MIR2 contribute critically to target selectivity (unpublished data). To better understand which regions of the target might contribute to MIR recognition, we took advantage of the fact that MIR2 can downregulate human but not murine MHC-I chains, even when the latter are expressed in a human cell (Fig. 7 A; Stevenson et al., 2000). Since both human and mouse MHC-I proteins contain cytosolic lysines, this suggests that the failure of MIR2 to regulate mouse MHC-I might reside at the level of target recognition. To further understand this issue, we constructed chimeras between mouse MHC-I (H2 Dd) and human MHC-I (HLA.B7) and tested their ability to be regulated by MIR2. BJAB cells stably expressing MIR2 or vector alone were transiently transfected with an expression vector for wt HLA B7 (which is not expressed endogenously by BJAB cells). All transfections proceeded with comparable effi-
ciency (unpublished data). As shown in Fig. 7 A, a 15-fold decrease in the number of cells expressing HLA B7 was observed in cells expressing MIR2 compared with cells expressing the control vector. As expected, mouse MHC-I chains did not display this MIR2-mediated downregulation. Similar experiments were performed using the chimeras depicted in Fig. 7 B. As shown in Fig. 7 B, chimera 3, containing the TM and intracellular region of mouse MHC, cannot be downregulated by MIR2. On the other hand, chimera 2, which differs from chimera 3 principally in containing the TM and juxtamembrane region of human MHC, is efficiently downregulated by MIR2. This suggests that the TM and juxtamembrane regions of the target MHC chains contribute critically to recognition by MIR proteins.

**Discussion**

Our results establish that MIR-mediated downregulation of the cell surface molecules involved in immune recognition is mediated by ubiquitination of their cytosolic tails followed by their endocytosis and subsequent lysosomal degradation. The in vitro ability of MIR2’s PHD domain to promote a Ub transfer reaction in an ATP- and E2-dependent fashion strongly indicates that the MIR proteins participate in this process by serving as E3 Ub ligases. The MIR proteins thus represent a novel class of membrane-bound E3 enzymes and are the first E3s to be found to be dependent on a Zn finger of the PHD rather than the RING subfamily. In this connection, it is of note that the first solution structure of a PHD domain has been published recently and reveals a striking structural relatedness to known RING fingers (Capili et al., 2001).

Although ubiquitination is associated most prominently with proteosome-mediated degradation, recent studies reveal roles for this modification in a variety of other processes in cell biology, including transcription factor function and membrane protein trafficking (Hicke, 2001b). Clear evidence links ubiquitination to the endocytosis of many proteins in yeast. In mammalian cells, this modification has been implicated in more specialized endocytic events, principally those involved in the ligand-induced downregulation of several growth factor receptors (Hicke, 2001a). In that setting, ubiquitination of the target is usually catalyzed by the RING finger-containing E3 known as cbl, which is recruited to the activated (tyrosine-phosphorylated) growth factor receptors via its SH2 domain. Ubiquitination is also
implicated in the endocytosis of the epithelial sodium channel (Enac); however, in this case the responsible E3 (Nedd4) is a member of the structurally unrelated HECT domain-containing family of E3s (Staub et al., 1997). Our data reveal that ubiquitination can also regulate the endocytosis of another class of cell surface targets, those involved in CTL recognition and helper T cell activation, and raise the possibility that this modification may be more widely involved in regulating the trafficking of other classes of targets. Indeed, although the initial identification of the MIR proteins was based on screens for viral genes that affect immune recognition, our finding that these proteins are actually E3 Ub ligases raises the question of whether they might function more broadly in the regulation of other Ub-dependent processes. Based upon this notion, searches for additional targets of MIR2-mediated ubiquitination are now underway.

In this connection, we note that a homologue of MIR1 has been discovered recently in a murine gammaherpesvirus, MHV68 (Stevenson et al., 2000). Unlike the KSHV MIR proteins, the MHV68 protein (called MK3) does not influence MHC-I endocytosis; rather, it functions to trigger MHC-I degradation. While this paper was being prepared for submission, a report appeared that revealed that this degradation could be inhibited by inhibitors of the proteasome and that in the presence of such inhibitors ubiquitinated MHC chains accumulated (Boname and Stevenson, 2001). In those studies, the authors could not detect interactions of MK3 with known components of the ubiquitination machinery and thus were unable to determine if the effects of MK3 on ubiquitination were direct or indirect. Our data strongly suggest that MK3 functions to directly recruit an E2 to murine MHC-I. How MHC chains ubiquitinated in the ER under the influence of MK3 are degraded by the proteasome remains to be clarified, but the process appears to differ in some respects from the previously described dislocation of MHC polypeptides from the ER to the cytosol mediated by cytomegalovirus proteins US2 and US11. Perhaps the process is initiated by digestion of the cytosolic tail of MHC-I by the proteasome, triggering degradation of the remainder of the chain by other proteolytic systems.

The findings of Boname and Stevenson (2001) on MK3 raise interesting questions concerning the diversity of mechanisms by which even related E3s can act. Ubiquitination by MK3 functions to trigger proteosome-directed proteolysis, whereas ubiquitination by the KSHV MIR proteins affects endocytosis and endolysosomal degradation. As noted previously, both MK3 and the KSHV MIR proteins are localized principally to the ER (Coscoy and Ganem, 2000; Stevenson et al., 2000). Consistent with this localization, MK3-directed degradation of MHC-I chains occurs predominantly in the ER. However, KSHV MIR1 and MIR2 do not impair the assembly or transport of target chains in the ER but exert their principal effects at the plasma membrane and the endocytic pathway. How this comes about is uncertain, but two possibilities can be envisioned. The first is that ER-bound MIR chains might direct the ubiquitination of their targets as they transit through the ER with the modified target proteins undergoing enhanced endocytosis once they reach the surface. However, such a model is not in accordance with our previous pulse-chase analyses, which show that most chains exit the ER unmodified; moreover, MHC-I chains made in the absence of MIR proteins can still be downregulated after MIR expression (Coscoy and Ganem, 2000). Finally, the findings of Boname and Stevenson (2001) indicate that if Ub were added to MHC-I in the ER, the likely consequence would be degradation. For these reasons, we favor the model that a small percentage of MIR chains escape the ER and reach the plasma membrane where they can serve their targeting function.

The basis of this targeting function has been illuminated by experiments (Fig. 7; D. Sanchez et al, personal communication), implicating the TM and juxtamembrane regions of both target and effector chains in target selection. Based on these studies, we propose a model for how MIR-mediated ubiquitination might proceed (Fig. 8). In this model, the TM and juxtamembrane regions of MIR2 and its target chains mediate an interaction in the plane of the membrane; this juxtaposes the NH2-terminal PHD domain of MIR2 with its associated E2 with the cytosolic tail of the target, thereby facilitating ubiquitination of its substrate lysine residues. How ubiquitination of the target promotes its endocytosis and endolysosomal degradation remains a matter for further study. In other systems, ubiquitination is known to affect both internalization and the delivery of endosomal contents to lysosomes (for review see Hicke, 2001a). The fact that coexpression of MIR2 with dynamin dominant negative mutants leads to accumulation of cell surface MHC-I (Coscoy and Ganem, 2000) suggests that MIR-induced ubiquitination likely targets at least the internalization step, but further work will be needed to determine if downstream events are also influenced by MIR2.

The seemingly paradoxical finding (Fig. 1 B) that certain chimeras between B7.1 and B7.2 can be downregulated by MIR1 (which cannot downregulate either wt B7 species) can
now be understood in terms of this model. Since wt B7.1 lacks cytosolic lysines (Fig. 5 A), it cannot be regulated by either MIR1 or MIR2 (Fig. 1 B, lines 1 and 8). However, a chimera containing only the TM region of B7.1 (Fig. 1 B, line 3) can be regulated by both MIR proteins. This suggests that both MIR1 and MIR2 can recognize the TM region of B7.1; their failure to regulate wt B7.1 is based primarily on their inability to ubiquitinate B7.1 cytoplasmic tail. The failure of MIR1 to regulate a chimera with the TM and intracytoplasmic regions of B7.2 indicates that the inability of MIR1 to regulate wt B7.2 is due to its inability to recognize the TM region of B7.2.

We do not yet know if MIR1 and MIR2 act alone or in concert with other as yet unidentified partners to recruit the ubiquitination machinery. However, the observation that KSHV MIR2 is inactive in murine cells (unpublished data) suggests that additional host proteins are likely involved in MIR-mediated downregulation. This would not be surprising, since many E3s operate in large multisubunit complexes (Jackson et al., 2000). Another reason to suspect roles for other host proteins in MIR function derives from the comparison of MK3 with MIR1 and MIR2. If ER-localized chains of one member of this protein family (MK3) can mediate ubiquitination of MHC-I in situ, why is this activity not observed for other members of the family? One possibility is that, in the case of the MIRs, host ER or cytosolic proteins may negatively regulate their E3 activity in this organellae. Finally, it will be important to clarify which of the many cellular E2s is/are the natural ligand(s) of MIR2. Although UbcH5a was active in the in vitro Ub transfer reaction orchestrated by MIR2's PHD domain, this result need not imply that this E2 is a true in vivo ligand of MIR2. Indeed, preliminary yeast two-hybrid analyses suggest that other E2s may be more likely candidates for this role. The identification of these and other host components that interact with MIR proteins should shed light on the molecular details of the connection between ubiquitination and endocytosis and further our understanding of this unusual pathway of immune evasion.

Materials and methods

Cell lines and cell culture

HeLa cells and Phoenix cells were grown in DME-H21 supplemented with 10% (vol/vol) FCS and penicillin streptomycin. BJAB lymphoma cells were grown in RPMI 1640 supplemented as described above.

Antibodies

For FACS® analysis, all antibodies were used at a concentration of 1 µg/106 cells. Anti-Ub monoclonal antibody (Santa Cruz Biotechnology, Inc.) was used for Western blot at a dilution of 1/1,000. Purified monoclonal anti-human HLA class I antigen (W6/32) was from Dako. Purified monoclonal anti-B7.2 and PE-conjugated monoclonal anti-human HLA-B7 were from Pharmingen. Monoclonal anti-B7.1 was provided by Lewis Lanier (University of California, San Francisco, CA).

Plasmids

B7.1, B7.2, H2 Dd, and HLA B7 cDNA clones were provided by Lewis Lanier (University of California). All constructs were amplified by PCR amplification using these cDNAs and cloned into BamHI and SalI restriction sites of pB MN2-Zin with the exception of the MIR1-EGFP and MIR2-EGFP fusion molecules. These fusions were cloned into pB¥, a modified pBMN vector in which the gene for neomycin phosphotransferase gene has been replaced by the puromycin-N-acetyl-transferase gene. The retroviral vector pMX-pie has been provided by Lewis Lanier (University of California). This vector expresses EGFP, and the puromycin N-acetyl-transferase gene and was used as a control for experiments involving pB¥ retroviral vectors.

The first 249 nucleotides of MIR2 coding for the PHD domain were amplified by PCR using oligonucleotides containing additional EcoRI and NotI sites. GST fusion proteins were created by cloning this PCR fragments into the pGEX 4T-1 vector (Amersham Pharmacia Biotech). As a control, a second vector was generated where MIR cysteine codons in position 56 and 59 were mutated to serine codons.

Retroviral infection

On transfection with the retroviral vectors (pB MN based, pBP based, or pMX-pie), the Phoenix packaging cell line produces replication-defective viral particles that can be used for stable expression in HeLa or BJAB cells. Phoenix cells were transfected, and the virus-containing supernatant was harvested 48 h after the transfection, filtered through a 0.45-µm filter, and diluted with Polybrene (8 µg/ml final dilution). HeLa cells and BJAB cells (in 6-well dishes) were infected by spin infection (800 g for 2 h at 20°C) using 2 ml of viral supernatant. Selection of pB MN-transduced cells was started 36 h after infection by adding 1 mg/ml of G418 for HeLa cells or 1.5 mg/ml of G418 for BJAB cells. Cells transduced with pMX-pie or pB¥ vectors were selected in the presence of 1 µg/ml of puromycin.

Flow cytometry analysis

Adherent cells were detached using enzyme-free/PBS-based cell dissociation buffer (GIBCO BRL) according to the manufacturer's instructions. Cells were washed in PBS/1% BSA and incubated with specific monoclonal antibodies (1 µg/106 cells) for 30 min at 4°C. Unconjugated bound mouse antibodies were revealed by a R-phycocerythrin-conjugated goat anti-mouse antibody. Cell surface fluorescence was analyzed with a Becton Dickinson FACScalibur®.

Immunoprecipitation and Western blotting

10-cm dishes of HeLa cells were scraped, and protein lysates were made by resuspending in RIPA buffer (for B7.1 and B7.2 immunoprecipitation) or PBS with 1% NP-40 (for MHC-I immunoprecipitation). The lysates were cleared of debris and incubated with 2 µg antibody for 1 h before addition of protein A/G+ agarose beads (Santa Cruz Biotechnology, Inc.). Immunoprecipitated proteins were separated on a polyacrylamide gel, transferred to nitrocellulose, and Western blotted with mouse anti-Ub, and then incubated with goat anti-mouse Ig HRP and visualized with Luminol (Santa Cruz Biotechnology, Inc.).

Protein purification

GST fusion proteins were expressed in log-phase E. coli Top 10 (Invitrogen) induced with 2 mM isopropyl β-D-thiogalactoside for 3 h at 37°C. Bacterial pellets were washed in PBS, suspended in 3 ml lysis buffer (50 mM Tris, pH 8, 120 mM NaCl, 3 mg/ml lysozyme, and protease inhibitors) and incubated on ice for 15 min. Cells were then lysed by addition of NP-40 (1% final concentration) and three freeze-thaw cycles. Lysates were clarified by centrifugation (12,000 g for 30 min) and stored at −80°C. GST fusion proteins were purified by incubating the lysates with GST followed by extensive washing of the GST. Quantification of the fusion proteins was determined by Bradford assay and Coomassie blue using BSA as standards.

In vitro ubiquitination assay

Ubiquitination assays, unless indicated, were performed for 90 min at 30°C in 50 µl of ubiquitination reaction buffer (50 mM Tris, pH 7.4, 2.5 mM MgCl2, 0.5 mM DTT, and 10 mM ATP) using 10 µg GS-bound GST fusion proteins (see above), 40 ng Ub-activating enzyme E1 (Calbiochem), 2 µg of His6 Ub (A.G. Scientific), and 1 µg of UbcH5a (A.G. Scientific). GS-bound materials were purified by extensive washing before Western blot analysis.

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