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Folding of an MHC class II-restricted tumor antigen controls its antigenicity via MHC-guided processing

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CD4$^+$ and CD8$^+$ T cell responses to endogenous retroviral envelope glycoprotein gp90 generate protective immunity to murine colon carcinoma CT26. A panel of I-A^d-restricted T cell hybridomas recognize gp90 synthesized by CT26 cells but not by other gp90-expressing tumors. Here we report that antigenicity resides in an incompletely folded form of gp90 that is unique to CT26. In contrast to more compact forms of gp90 that are present in other tumors, this open conformer is captured by recycling I-A^d on antigen-presenting cells and is processed intracellularly. Thus, gp90 acquires immunodominance via MHC-guided processing, and the generation of an MHC class II-restricted response can be controlled by the intracellular folding environment of antigen-expressing cells.

antigen presentation | immunodominance | protein folding

Two antigen-processing pathways have been described for the loading of MHC class II molecules (MHC-IIs) with peptides for presentation to CD4$^+$ T cells. In the classical pathway, exogenous antigens taken up by antigen-presenting cells (APCs) are unfolded and cleaved into fragments during transport through the increasingly acidic endosomal network, from early endosomes to lysosomes. Newly synthesized MHC-IIs associated with the invariant chains (Iis) are transported to a late endocytic compartment where the Ii is removed by proteolytic cleavage, leaving the class II Ii-derived peptide (CLIP) bound to the peptide-binding groove (1, 2). This is followed by peptide editing, which is catalyzed by the MHC-encoded DM molecule (3–6). The initial forms of antigen that bind to MHC-IIs may be short peptides, 12–26 aa in length, generated by lysosomal proteases (7, 8). A second pathway has been described in which mature MHC-IIs recycle from the cell surface to early endosomes and load antigenic peptides that are made available in less acidic and less aggressively proteolytic environments, independently of newly synthesized MHC-IIs, Iis, and DM molecules (9–12).

A long-standing question concerning antigen processing in the MHC class II pathway is whether peptides are always generated first and then loaded (trim/bind model) or whether epitopes can be generated after the binding of intact antigen to MHC-IIs (bind/trim model) (13–16). The former model has been presumed to prevail, but Sercarz *et al*. (13, 14) have suggested that immunodominant epitopes within an intact, partially unfolding antigen might first bind to MHC-IIs and subsequently be trimmed to short peptide epitopes that roughly corresponded to the "footprint" of the MHC binding site. This latter model, or MHC-guided processing, has its roots in reports of MHC-IIs binding to long polypeptides (17–19). It remains unclear, however, whether these large polypeptides were the precursors of immunogenic peptides or whether they were presented as large polypeptides. The structure of several MHC-II/peptide complexes has now been solved, and it seems likely that peptides preferentially interact with the peptide-binding groove in an extended conformation, although some "bulging" is permitted for certain MHC-II/peptide combinations (20, 21). These data preclude the binding of MHC-IIs to elements of a protein antigen containing significant secondary structure. One mechanism by which large proteins or polypeptides bind to MHC-IIs might therefore be solvent-exposed regions of eight or more amino acids that adopt an extended or random conformation. Several proteins are known to display a more "relaxed" conformation during either thermal or chemical denaturation or during biogenesis. The latter may therefore give rise to a pool of substrates capable of binding to MHC-IIs and engaging in MHC-guided processing.

Here we describe how the notion of MHC-guided processing is related to the immunodominance of a recently identified MHC class II-restricted tumor antigen, murine leukemia virus (MuLV) envelope protein gp70/90 from mouse colorectal tumor CT26 cells (22). gp90 is synthesized in the endoplasmic reticulum in many mouse tumor lines as a precursor protein before cleavage in the Golgi apparatus to form the extracellular glycoprotein gp70 and transmembrane protein p15E (23–25). We have found that only gp90 synthesized by CT26 stimulates a panel of CD4$^+$ T cell hybridomas generated by the immunization of syngeneic BALB/c mice with CT26 (22). In contrast, gp90 from other mouse tumors is not stimulatory. Furthermore, the antigenicity of gp90 correlates with binding to the lectin-like chaperone calreticulin (CRT) and is strictly dependent on its conformation, namely, denaturation and unfolding abrogate recognition of gp90 by all of the established tumor-specific T cell hybridomas (22). Here we investigate the molecular basis for the conformation-dependent and tumor-specific antigenicity. We show that gp90 consists of multiple folding species including a CRT-bound incompletely folded conformer that is responsible for antigenicity. Conformation-dependent antigenicity results from selective binding of this conformer to recycling I-A^d MHC-IIs and intracellular processing, i.e., via a bind/trim pathway.

Results

The Antigenic Form of gp90 Is a Conformational Variant. The *env* gene product gp90 of murine leukemia virus (MuLV) was detected as a single band migrating at 90 kDa by reducing SDS/PAGE from three BALB/c-derived colon tumor lines (Fig.

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The authors declare no conflict of interest.

Abbreviations: MHC-II, MHC class II molecule; APC, antigen-presenting cell; Ii, invariant chain; CRT, calreticulin; OVA, ovalbumin.

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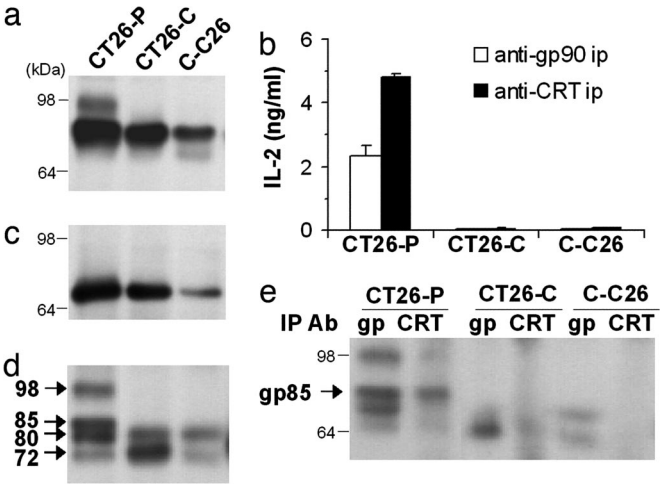

Fig. 1. Incompletely folded gp90 conformer from CT26-P is antigenic. (*a*) Western blot of reduced gp90 isolated by anti-gp90 immunoprecipitation from the lysates of 1×10^6 tumor cells. (*b*) IL-2 release from CT26-specific hybridoma 5 cells cocultured with A20 cells prepulsed with anti-gp90 (open bars) or anti-CRT immunoprecipitates (ip) (filled bars) of the lysates of 1×10^6 tumor cells. The same quantity of samples used in the Western blot shown in *a* was analyzed in the IL-2 assay. The data are shown as the mean with SE of two independent experiments. (*c*–*e*) Western blots of gp90 deglycosylated with peptide: N-glycosidase F (PNGase F) (*c*), nonreduced gp90 (*d*), and gp90 coprecipitated with CRT (*e*). IP Abs, immunoprecipitating Abs; gp, anti-gp90 Abs; CRT, anti-CRT Abs.

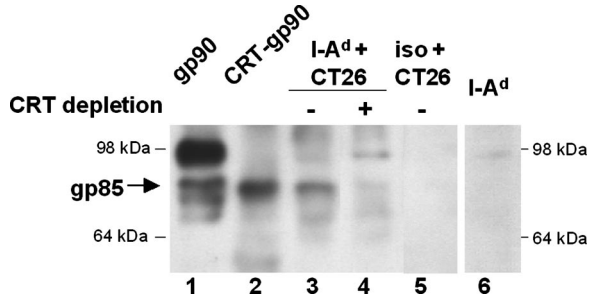

Fig. 2. Specific binding of I-A^d to gp85. I-A^d immunoprecipitate from A20 cells specifically bound to gp85 in the CT26-P cell lysate (lane 3). Note that the I-A^d gp85 coimmunoprecipitate was not observed with CRT-depleted lysate (lane 4). Isotype-matched Ab (iso) with anti-I-A^d (IgG2a κ) (lane 5). The Western blot was probed with anti-gp70/90 goat antiserum.

1*a*). Two of these, CT26-P and CT26-C, are reported to be genetically identical (26). Interestingly, only gp90 from CT26-P cells stimulated IL-2 production from the tumor-specific T cell hybridoma (Fig. 1*b*) despite the identical nucleotide sequence of gp90 from all three lines [supporting information (SI) Fig. 7]. Consistent with this, gp90 from all three cell lines migrated as a single major 90-kDa band during reducing SDS/PAGE and resolved as a single 70-kDa band upon deglycosylation with peptide: N-glycosidase F (PNGase F) (Fig. 1*c*). CT26-P cells uniquely expressed an additional 98-kDa species (Fig. 1*a*), which was identified as a gp90 variant bearing complex-type glycans because it was resistant to endoglycosidase H (endo H) digestion (SI Fig. 8). In contrast, the main gp90 band was sensitive to endo H and was shown to have high-mannose-type N-glycans by HPLC analysis (SI Figs. 8 and 9). Importantly, when fractionated by nonreducing SDS/PAGE, only the gp90 from CT26-P cells was resolved into three bands and uniquely contained a slower migrating or "floppy" species of 85 kDa (hereafter referred to as gp85), whereas gp90 from other cell lines was present as two compact forms migrating at 80 and 72 kDa (Fig. 1*d*). We have shown previously that gp90 antigenicity correlates with CRT binding (22), so we next examined which of the conformers identified by nonreducing SDS/PAGE was complexed to CRT by coimmunoprecipitation. Only the floppy conformer, gp85, from CT26-P cells was coprecipitated with CRT (Fig. 1*e*). The CRT-bound gp85 was observed only in the product from CT26-P cells and was antigenic (Fig. 1*b*), suggesting that T cell recognition of gp90 is conformation-dependent and that the floppy 85-kDa form is responsible for antigenicity. In addition to CRT binding, we have investigated the susceptibility of gp90 to proteases as a surrogate marker for conformational difference and shown that limited digestion of gp90 from CT26-P and CT26-C with V8 protease gives rise to different patterns of degradation products (data not shown). Furthermore, the binding of I-A^d MHC-IIs to the gp90 conformers was examined. When I-A^d immunoprecipitate was mixed with the CT26-P lysate, only gp85 was coprecipitated (Fig. 2, lane 3). I-A^d-bound gp85 was not detected when a CRT-depleted lysate of CT26-P cells was used (Fig. 2, lane 4). Therefore, these results demonstrate that the gp85 conformer represents the major active component of gp90 and is uniquely selected as a substrate by CRT.

MHC-Guided Processing of gp90. To examine whether the conformational features of gp90 that determine its antigenicity might reside in its ability to bind directly to I-A^d at the cell surface without processing (the bind/trim sequence), we constructed an antigen presentation assay that mimics the bind/trim model, using glutaraldehyde-fixed APCs and trypsin digestion (Fig. 3*a*). Ovalbumin (OVA), used as a control, was presented to an OVA-specific hybridoma when it was digested with trypsin before incubation with fixed APCs (trim/bind) but not after (Fig. 3*b Left*). In contrast, gp90 from CT26-P was not stimulatory after proteolysis (Fig. 3*b Left*). However, gp90 was presented by fixed APCs to all of the three CT26-specific T cell hybridoma clones when tryptic digestion was performed after incubation with fixed APCs (bind/trim) (Fig. 3*b Center*). Importantly, only gp90 from CT26-P cells stimulated IL-2 production: gp90 from CT26-C cells did not (Fig. 3*b Right*). CRT-bound gp90 from CT26-P was also presented by the bind/trim model when offered to APCs as a complex (data not shown). Inhibition of gp90 presentation was shown with anti-I-A^d (Fig. 3*c Left*) and anti-gp70/90 (Fig. 3*c Center*) Abs and the OVA peptide (323–339) (Fig. 3*c Right* and ref. 27). Thus, gp90 is an example of a naturally occurring protein antigen for which a specific conformer can be presented by the bind/trim model. The results suggest that, when offered to live APCs, gp90 is presented by mature MHC-IIs after capture and subsequent processing at the cell surface or after recycling through early endocytic vesicles.

Neither Deglycosylation nor Reduction/Alkylation Compromises gp90 Presentation by Fixed APCs. We examined whether the differential antigenicity between the gp90 conformers is due to posttranslational modification of the CD4$^+$ T cell epitope. Because gp90 is a glycoprotein in the early secretory pathway, as evidenced by the presence of monoglucosylated high-mannose-type N-glycans (see SI Fig. 9 and Table 1), we assumed that the most likely candidates for posttranslational modification that can result in differential folding and CRT binding in the endoplasmic reticulum could be N-glycosylation or disulfide bond formation. However, the T cell response to deglycosylated gp90 from CT26-P cells was slightly higher than the mock-digested gp90, whereas gp90 from CT26-C cells was not stimulatory, regardless of glycosylation status (Fig. 4*a*), indicating that the T cell determinant of gp90 is not dependent on N-glycosylation for either its generation or recognition. The slightly increased antigenicity of gp90 that we observed after deglycosylation may

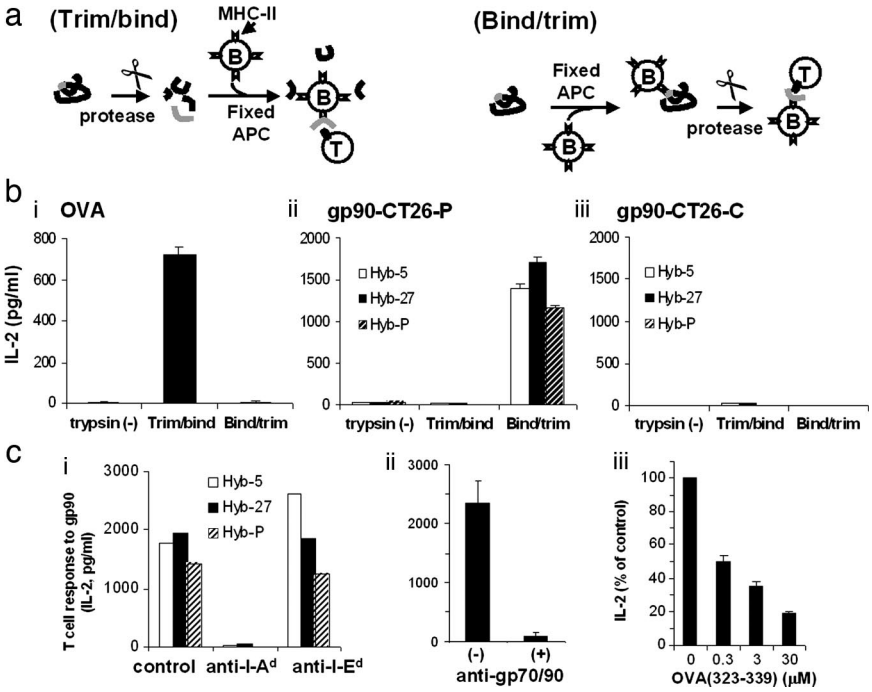

Fig. 3. gp90 is presented by the bind/trim model. (*a*) Diagram of the presentation assay to test the trim/bind and bind/trim models when using fixed A20 cells and digestion with trypsin. B, antigen-presenting B cell; T, T cell hybridoma. (*b*) Presentation by fixed A20 cells of OVA (*Left*), gp90 from CT26-P (*Center*) or CT26-C cells (*Right*). (*c*) Inhibition of gp90 presentation by fixed APCs with anti-I-A^d or I-E^d Ab (*Left*), anti-gp70/90 serum 80S24 (*Center*), and OVA peptide (323–339) (*Right*). The data are shown as the mean with SE of two independent experiments. Hyb, hybridoma.

result from increased accessibility of the epitope to I-A^d. In a separate approach, we identified all of the I-A^d-binding peptides among a library of 15-mers (overlapping by 10 aa) spanning the entire gp90 sequence that contained an NXT/S motif and tested them for their ability to stimulate T cell hybridomas, but none were active (SI Fig. 10).

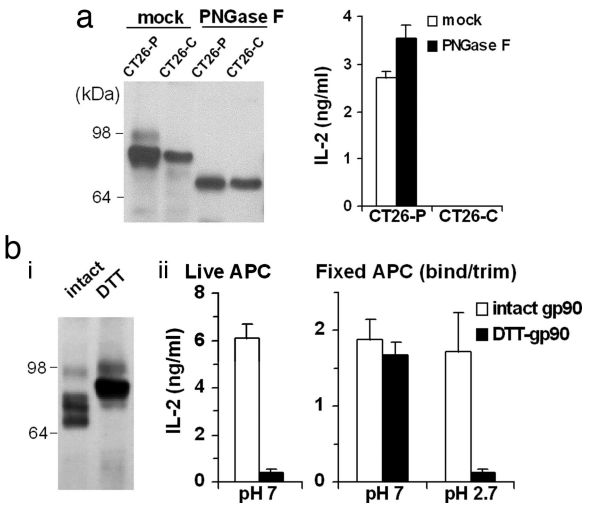

Fig. 4. gp90 presentation by fixed APCs is insensitive to deglycosylation or reduction/alkylation. (*a*) Western blot of deglycosylated gp90 and IL-2 production by hybridoma 27 cocultured with fixed A20 cells that were prepulsed with glycosylated or deglycosylated gp90 on ice for 8 h and trypsinized. PNGase F, peptide: N-glycosidase F. (*b*) Western blot of reduced and alkylated gp90 (DTT gp90) (*Left*) and presentation of DTT gp90 by live or fixed APCs (*Right*). Intact gp90 and DTT gp90 were present in gp90 immunoprecipitates in PBS (pH 7) or eluted from immunoprecipitates with acidic glycine/HCl buffer and neutralized (pH 2.7). The data are shown as the mean with SE of two independent experiments.

The influence of sulfhydryl group modification on gp90 antigenicity was tested with reduced and alkylated gp90 from CT26-P cells (DTT gp90, Fig. 4*b Left*). As reported previously (22), reduction and alkylation can abrogate antigenicity of gp90 when pulsed to live APCs (Fig. 4*b Right*). However, DTT gp90 in PBS (pH 7) retained its antigenicity with fixed APCs in the bind/trim presentation assay (Fig. 4*b Right*, pH 7), indicating that modification(s) of cysteine residues are not involved in forming the gp90 epitope. Importantly, DTT gp90 that was unfolded and refolded by exposure to acidic glycine buffer and then neutralization (Fig. 4*b Right*, pH 2.7) was no longer antigenic in contrast to the intact gp90. This suggests that the difference in the stability between intact and reduced gp90 accounts for the diminished antigenicity of reduced gp90 when offered to live APCs.

gp90 Presentation via the Recycling MHC Class II Pathway. We investigated this bind/trim mechanism in more detail by treating living APCs with compounds that are known to block the classical presentation pathway: leupeptin, which inhibits cathepsin S and Ii processing; chloroquine, a nonspecific inhibitor of acid proteases; bafilomycin A1, which inhibits vesicular transport from early endosomes to late endosomes; and MV026630, which inhibits the early processing enzyme asparagine endopeptidase (28). We also investigated the effect of brefeldin A, which prevents the transport of newly synthesized MHC-IIs from the endoplasmic reticulum and disrupts traffic between endosomes and lysosomes while maintaining normal cycling between the plasma membrane and endosomes (29). The efficacy of the compounds was confirmed by their ability to inhibit the presentation of OVA. None of these pharmacological interventions blocked gp90 presentation (Fig. 5*a*). Interestingly, although brefeldin A inhibited the presentation of OVA by ≈40%, it enhanced presentation of gp90 by >3-fold. The increase in the presentation of gp90 with brefeldin A may result from more efficient trimming of the antigen bound to MHC-IIs by endo-

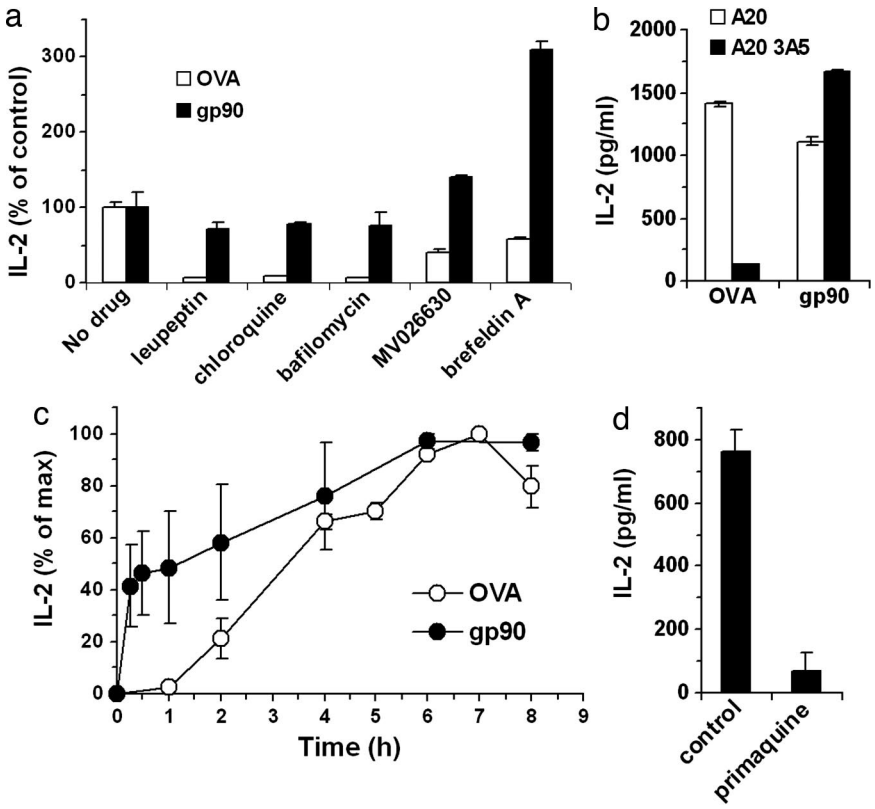

Fig. 5. Comparison of presentation pathway between OVA and gp90. (*a*) Effects of drug treatments on presentation of OVA (open bars) and gp90 (filled bars). (*b*) Presentation of OVA and gp90 by DM-deficient mutant A20 3A5. (*c*) Kinetics of OVA and gp90 presentation by A20 cells. (*d*) Effect of primaquine on gp90 presentation. The data are shown as the mean with SE of two independent experiments.

somal proteases by suppressing its exposure to destructive processing in the lysosomes.

We also investigated the H2-DM dependence of gp90 presentation (3–6). When the DM-deficient cell line A20 3A5 (30) was used, OVA presentation decreased by 90%, but gp90 presentation was slightly enhanced (Fig. 5*b*). This increase is consistent with an earlier report showing enhanced presentation of an albumin epitope presented by recycling MHC-IIs in APCs lacking DM (31). The involvement of recycling MHC-IIs in gp90 presentation was further supported by the faster kinetics of presentation of gp90 compared with that of OVA: gp90 presentation was detectable within 15 min of pulsing APCs, whereas OVA presentation was not significant before 2 h (Fig. 5*c*). This is consistent with a previous report that recycling of MHC-IIs reaches a plateau within 10–15 min (10). Last, we examined the effect of primaquine, which has been reported to allow cell surface endocytosis but to block the cycling of receptors and MHC molecules back to the plasma membrane (32, 33), although its molecular target has not been identified. Primaquine was found to abrogate gp90 presentation by 90% (Fig. 5*d*), suggesting that the floppy form of gp90 requires intracellular processing rather than proteolysis at the cell surface for presentation by recycling, mature MHC-IIs. Taken together, these data support the operation of an MHC-guided antigen-processing pathway that requires proteolysis in early endocytic compartments.

Discussion

The original studies of T cell recognition of protein antigens indicated that recognition of denatured antigens was as efficient as that of native antigens (34); therefore, it has been assumed that antigen conformation is not an important factor in determining antigenicity. In contrast, the immunodominant MHC class II-restricted tumor antigen gp90 exhibits conformation-dependent and tumor-specific antigenicity (22). The $CD4^+$ T cell response to a specific antigenic folding conformer of gp90 was induced upon immunization of mice with a whole CT26 cell vaccine. Here we link protein antigen folding in a natural environment to antigenicity and provide a mechanistic framework for understanding this process.

There have been reports that artificially unfolded antigens can bind to MHC-IIs and be presented (35); however, it should be noted that the floppy and compact forms of gp90 are naturally occurring conformers that presumably arise in different cellular environments, and that the unfolding of gp90 abrogates its antigenicity rather than promoting it (22) (Fig. 4*b*). Our antigen presentation assay showed that the T cell response to gp90 was not observed without tryptic digestion after binding to MHC-IIs (Fig. 3*b Center*). It therefore differs from previous reports concerning the presentation of large unfolded proteins such as fibrinogen, whose T cell epitope at the C terminus can be presented by fixed APCs without processing (36). The antigenicity of gp90 is exquisitely sensitive to prior proteolysis, with trypsin (Fig. 3*b Center*), chymotrypsin, V8 protease, and cyanogen bromide abrogating its presentation (data not shown). It seems unlikely, therefore, that extensive proteolysis would precede MHC binding in the natural processing of this antigen. Epitopes that are generated via the alternative bind/trim pathway may contain a remnant of secondary structure or "bulge" in the floppy conformer of the whole-protein antigen. This may explain why we were unable to identify an epitope from extensive peptide libraries of 15 aa overlapping by 10 aa, including synthetic glycopeptides (37) (data not shown). Indeed, recent reports of T cell receptors specific for a bulged peptide/MHC-I or MHC-II complex are in agreement with this idea (20, 21).

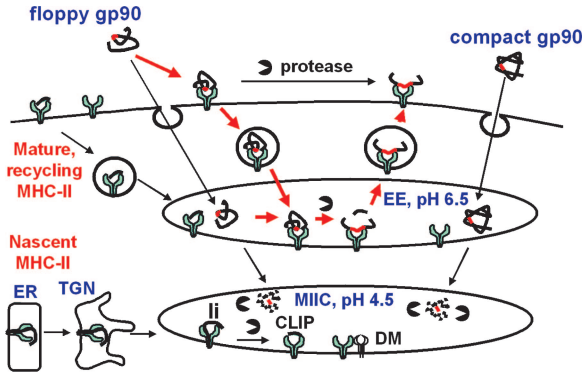

Fig. 6. Summary of presentation of differential gp90 folding conformers by MHC-IIs. The recycling pathway is shown by red arrows. MHC-IIs bind an exposed loop of floppy gp90 at the cell surface. The complex is internalized into early endosomes (EE), where further processing occurs. This process is mimicked when extracellular proteases act on gp90 bound at the surface of APCs. Similarly, recycling MHC-IIs may encounter native gp90 in early endosomes and bind before being returned to the cell surface. Compact gp90 does not bind to MHC-IIs at the cell surface or in the early endosomes, probably because the epitope is not exposed and is transported to the MHC class II compartment (MIIC) where the epitope is destroyed by overprocessing with proteases. TGN, transGolgi network.

Presentation of gp90 by recycling MHC-IIs is evidenced by the insensitivity of its processing to leupeptin, chloroquine, and bafilomycin A1 and sensitivity to primaquine and is consistent with DM independence. The lack of involvement of the classical pathway in gp90 processing may be due to overprocessing of the epitope and/or the inability of unfolded gp90 peptide fragments to bind to MHC-IIs at low pH in late endocytic compartments (SI Fig. 11). The epitope of OVA is loaded onto MHC-IIs in late endosomal compartments/MHC class II compartments in a DM-catalyzed process, whereas, for fully folded, compact gp90 synthesized by CT26-C cells, its entry into this pathway results in the destructive processing of the immunodominant I-A^d-restricted epitope (summarized in Fig. 6). The contribution of the recycling MHC class II pathway to the overall level of MHC class II-restricted antigen processing is unclear, although presentation by recycling MHC-IIs has been reported for other proteins including influenza virus hemagglutinin (9, 10), myelin basic protein (10), and ribonuclease (38). We have shown that recycling MHC-IIs can capture a specific conformer of an antigen at the surface or early endosomes of APCs.

To conclude, the biological significance of this finding is that the intracellular folding environment of a protein could be as important as its primary structure or posttranslational modification in determining its antigenic properties for T cells. Thus, differential processing/presentation of the same protein can occur in APCs, depending on the folding status of the protein, which has relevance to antitumor immunity and autoimmune responses to misfolded self-proteins.

Materials and Methods

Cell Lines. The CT26 murine colon tumor line was originated by intrarectal injections of *N*-nitroso-*N*-methylurethane into BALB/c mice (26), with CT26-P provided by D. M. Pardoll (The Johns Hopkins University, Baltimore, MD) (39) and CT26-C by M. Colombo (University of Milan, Milan, Italy) without any genetic modification. C26 is a BALB/c-derived colon carcinoma cell line (40). A20 B lymphoma cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). The H2-DM-deficient mutant of A20 (A20 3A5) and OVA-specific T cell hybridoma DO-11.10/S4.4 were generous gifts of K. L. Rock (University of Massachusetts Medical School, Worcester, MA) and P. Marrack (National Jewish Medical and Research Center, Denver, CO), respectively. CT26-P, C-C26, A20, A20 3A5, and DO-11.10/S4.4 were cultured in RPMI medium 1640 supplemented with 10% FBS, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 50 μM mercaptoethanol. CT26-C was maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. CT26-specific $CD4^+$ T cell hybridomas, hybridomas 5, 27, and P, were established in our laboratory (22). Please see *SI Materials and Methods* for further details.

Abs. Anti-gp90 (or p15E) (372) mouse IgG3 hybridoma and anti-I-A^d mouse IgG2a κ hybridoma (MK-D6) were purchased from ATCC. The mAbs were purified on protein G Sepharose 4B (Amersham Biosciences, Piscataway, NJ) from supernatants of the hybridoma cultured with Hybridoma-SFM (Gibco, Carlsbad, CA). Anti-I-$E^{k,d}$ (14.4.4S), anti-gp70/90 goat serum (80S24), and anti-CRT polyclonal rabbit serum were purchased from Clontech (Mountain View, CA), National Cancer Institute/ViroMed Biosafety Laboratories (Minnetonka, MN), and Affinity BioReagents (Golden, CO), respectively.

Immunoprecipitation. gp90-expressing cells were washed with PBS once and lysed at 1×10^7 cells per milliliter of the pH 7 lysis buffer [10 mM Tris·HCl/0.15 M NaCl/1% Triton X-100/complete EDTA-free protease inhibitor cocktail tablets (Roche Applied Science, Indianapolis, IN), pH 7.0] at 4°C for 30 min. Cell lysate was centrifuged by using a Beckman Coulter (Fullerton, CA) TLA-100.3 rotor in a tabletop ultracentrifuge at 100,000 $\times$ *g* at 4°C for 5 min. The supernatants were precleared with protein G Sepharose 4B for 1 h and immunoprecipitated by using anti-gp90 (10 μg per 1×10^6 cell equivalents of cell lysate) or anti-CRT serum (3 μl per 1×10^6 cell equivalents of cell lysate), together with protein G Sepharose 4B (50 μl of 50% slurry per 1×10^6 cell equivalents of cell lysate) for 2 h. The immunoprecipitates were washed once with the pH 7 lysis buffer, once with 50 mM Hepes and 10 mM phosphate containing 0.5% CHAPS (pH 7.0), and once with PBS. They were then treated with SDS sample buffer with or without 5% 2-mercaptoethanol.

IL-2 Assay. IL-2 release assays were done with a mouse IL-2 ELISA kit (Endogen, Woburn, MA) by using culture supernatants of 1×10^5 specific T cell hybridomas cocultured with 2×10^5 A20 cells pulsed with antigen in a 96-well flat-bottom plate for 16 h.

Analysis of gp90 Binding to MHC-IIs. A20 cells (2×10^6) were lysed in 1 ml of the pH 8 lysis buffer (10 mM Tris·HCl/0.15 M NaCl/1% Triton X-100/complete EDTA-free protease inhibitor cocktail tablets, pH 8.0) for 30 min, precleared with protein G Sepharose 4B, and immunoprecipitated with 10 μg of anti-I-A^d Ab (MK-D6) for 2 h at 4°C. Isotype-matched Ab (anti-fluorescein 4-4-20, Molecular Probes, Carlsbad, CA) was used as a control. The I-A^d immunoprecipitate was washed with the pH 8 lysis buffer twice and the pH 7 lysis buffer once; it was then mixed overnight with lysate of 1.25×10^6 CT26-P cells prepared with the pH 7 lysis buffer or lysate that was precleared of CRT-bound gp85 by serial anti-CRT immunoprecipitation twice. The coimmunoprecipitate was washed with the pH 7 lysis buffer once, with 50 mM Hepes and 10 mM Na_2PO_4 containing 0.5% CHAPS (pH 7.0) once, and with PBS once, followed by treatment with SDS sample buffer for nonreducing SDS/PAGE.

Bind/Trim Presentation Assay. For the bind/trim assay, glutaraldehyde-fixed A20 cells (2×10^5 cells per well) were incubated with 100 μg of OVA (grade VI; Sigma, St. Louis, MO) or gp90 isolated by immunoprecipitation from 1×10^6 CT26 cells in Hanks' balanced salt solution (HBSS) (pH 7.0) in a 96-well

flat-bottom plate for 3 h at 37°C. The cells were washed with HBSS three times and treated with 20 μl of 0.5 mg/ml trypsin–EDTA (Gibco) at 37°C for 10 min. To inactivate trypsin, 2 μl of 20 mg/ml soybean trypsin inhibitor and 100 μl of fresh medium was added. The cells were centrifuged and resuspended in 100 μl of fresh medium and cocultured with 100 μl of specific T cell hybridoma (1×10^5 cells per well) for 16 h. For the trim/bind assay, fixed A20 cells were sensitized in HBSS at 37°C for 3 h with peptide fragments that were digested with 10 μg of trypsin at 37°C for 5 min and then with 40 μg of trypsin inhibitor, washed with HBSS twice, resuspended in 100 μl of fresh medium, and cocultured with specific T cell hybridoma.

Inhibition of gp90 presentation by fixed APCs with anti-I-A^d (MK-D6) and anti-I-E^d (14.4.4S) Abs was performed by incubation of gp90 pulsed and trypsinized APCs with each Ab (5 μg) for 30 min at 37°C. After washing with HBSS three times, the APCs were cocultured with the T cell hybridomas. The inhibitory effect of anti-gp70/90 Ab (80S24) on I-A^d binding was examined by preincubation of purified gp90 (0.1 μg) with the Ab 80S24 for 2 h, incubation with fixed APCs for 3 h, trypsinization, and coculture with T cell hybridoma 27 as described above. The I-A^d-restricted OVA (323–339) ISQAVHAAHAEINEAGR (CSS–Albachem, East Lothian, United Kingdom) was used as an inhibitor for the binding of gp90 to I-A^d at 0.3–30 μM. The cells were washed, trypsinized, and cocultured with hybridoma 27 cells as described above.

Kinetics of Antigen Presentation. A20 cells (2×10^5 cells per well) were pulsed with gp90 (anti-gp90 immunoprecipitate) prepared from 1×10^6 CT26-P cells or 100 μg of OVA in RPMI medium 1640 containing 10% FBS at 37°C for up to 8 h. For negative control (time 0), prefixed A20 cells were pulsed with the antigen. Antigen processing was terminated by fixation with 1% paraformaldehyde for 20 min. After washing with HBSS three times, with complete medium for 30 min at 37°C, and with HBSS twice, the fixed, antigen-loaded A20 cells were cocultured with specific T cell hybridoma (1×10^5 cells per well) for 16 h.

Drug Treatments. A20 cells (2×10^5 cells per well) were pretreated with 1 mM leupeptin (Roche, Gipf-Oberfrick, Switzerland), 0.4 mM chloroquine (Sigma), 0.1 μM bafilomycin A1 (Calbiochem, San Diego, CA), or 20 μM MV026630 (generous gift from C. Watts, University of Dundee, Dundee, United Kingdom) for 1 h or with 30 μM brefeldin A (Sigma) for 15 min at 37°C before the addition of gp90 or 100 μg of OVA. The A20 cells then were pulsed with antigen for 8 h and fixed with 1% paraformaldehyde for 20 min. After washing as described above, the A20 cells were cocultured with specific T cell hybridoma (1×10^5 cells per well) for 16 h. For primaquine treatment (Aldrich, Milwaukee, WI), A20 cells were pretreated with the compound at 0.2 mM for 30 min, pulsed with gp90 for 30 min to avoid cell death during the treatment, fixed, and cocultured with the T cell hybridoma.

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