MHC molecules bind antigenic peptides and present them to T cells. There is a growing body of evidence that MHC molecules also serve other functions. We and others have described synthetic peptides derived from regions of MHC molecules that inhibit T-cell proliferation or cytotoxicity in an allele-nonspecific manner that is independent of interaction with the T-cell receptor. In this report, we describe the mechanism of action of a synthetic MHC class II–derived peptide that blocks T-cell activation induced by IL-2. Both this peptide, corresponding to residues 65–79 of DQA*03011 (DQ 65-79), and rapamycin inhibit p70 S6 kinase activity, but only DQ 65-79 blocks Akt kinase activity, placing the effects of DQ 65-79 upstream of mTOR, a PI kinase family member. DQ 65-79, but not rapamycin, inhibits phosphatidylinositol 3-kinase (PI 3-kinase) activity in vitro. The peptide is taken up by cells, as demonstrated by confocal microscopy. These findings indicate that DQ 65-79 acts as an antagonist with PI 3-kinase, repressing downstream signaling events and inhibiting proliferation. Understanding the mechanism of action of immunomodulatory peptides may provide new insights into T-cell activation and allow the development of novel immunosuppressive agents.

**Methods**

**Peptides, antibodies, and reagents.** Peptides were prepared as described (15). Rapamycin was a gift from Wyeth-Ayerst Laboratories (Philadelphia, Pennsylvania, USA). Wort-
mannin was purchased from Calbiochem-Novabiochem Corp. (San Diego, California, USA). Ab’s specific to p70 S6 kinase, Fyn, and Lck were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Ab’s to phosphotyrosine (4G10), Jak1, Jak3, Fyn, Lck, Akt, p85, and p110-β were purchased from Upstate Biotechnology Inc. (Lake Placid, New York, USA). Ab’s specific to IL-2Rα, IL-2Rβ, and IL-2Rγ were purchased from PharMingen (San Diego, California, USA). Unless specified, all other reagents were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA).

Peripheral blood leukocyte and T-cell isolation and culture. Peripheral blood leukocyte isolation and T-cell purification were done as described (18). Cells were cultured in RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin/streptomycin, 2 mM L-glutamine, and 10 mM HEPES. IL-2–dependent T cells were prepared by stimulating T cells with phytohemagglutinin P (5 μg/mL) for 72 hours. The cells were then cultured with 100 U/mL IL-2. Once a week, the cells were restimulated with phytohemagglutinin P (0.8 μg/mL). In experiments using wortmannin, cells were cultured in AIM-V medium (Life Technologies Inc., Gaithersburg, Maryland, USA).

Immunoprecipitation and Western blot. IL-2–dependent T cells (1 × 10⁷ to 2 × 10⁷) were washed twice with PBS (pH 6.5) and incubated for 3 hours in RPMI 1640 containing 0.5% human AB serum. This was followed by incubation with peptide (40 μM), rapamycin (100 nM), or wortmannin (100 nM) for 30 minutes before stimulation with 1,000 U/mL IL-2 for 15 minutes. After treatment, cells were recounted, lysed, and subjected to immunoprecipitation and blotting as described (15).

Fyn kinase and Lck kinase assays. IL-2–dependent T cells (5 × 10⁶ per condition) were activated with IL-2 as described above. Cells were lysed and kinase assays were performed essentially as described (19).

Assay for p70 S6 kinase. IL-2–dependent T cells (10⁷ per condition) were activated with IL-2 in the presence of various agents as described above for immunoprecipitation. The samples were divided into two equal aliquots, lysed in buffer B (0.1% NP-40, 2 mM DTT, 50 mM β-glycerophosphate, 10 mM K2HPO4, 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2, 40 μg/mL PMSF, and 1 mM Na3VO4), and immunoprecipitated with anti-p70 S6 kinase. One aliquot was subject to SDS-PAGE and Western blotting as described above. The other aliquot was processed for kinase activity. The immunoprecipitated kinase was washed twice in buffer B and then washed twice in 10 mM β-glycerophosphate, 10 mM MgCl2, 10 μg/mL leupeptin, 0.4 mM DTT, and 20 mM Tris (pH 7.5). The kinase buffer (40 μL) contained 100 μM ATP, 200 μCi/mL [γ-32P]ATP (Amersham Life Sciences Inc., Arlington Heights, Illinois, USA) and 125 μM S6 peptide (Santa Cruz Biotechnology Inc.). The reaction mixture was incubated for 15 minutes at 30°C, and then the reaction was terminated by adding 40 μL of ice-cold 20 mM EDTA (pH 8.0). Triplicate aliquots (10 μL) were spotted onto phosphocellulose membranes (Life Technologies Inc.). Membranes were immersed briefly in a 1% H3PO4 solution containing 10 mM Na2P2O7, washed in 1% H3PO4, and quantitated by liquid scintillation counting.

Akt kinase assay. IL-2–dependent T cells (10⁷ per condition) were activated with IL-2 as described above. Akt kinase assay was performed using a kit from Upstate Biotechnology Inc.

Phosphatidylinositol 3-kinase assay. IL-2–dependent T cells (5 × 10⁶ per condition) were activated with IL-2 as described above. Cells were lysed, and the phosphatidylinositol 3-kinase (PI 3-kinase) assay was performed as described (20). For experiments in which the peptide was added directly to the kinase assay, cells were prepared without peptide treatment before immunoprecipitation. Either the peptide (40 μM), rapamycin (100 nM), or wortmannin (100 nM) was added to the kinase reaction at the same time as the lipid substrate.

Cell staining and confocal microscopy. A CD8⁺ long-term cytotoxic T-lymphocyte line (10⁶ cells/mL) was incubated in RPMI 1640 supplemented with DMSO or DQ 65-79–IRS (NIAVLKHNLIVIKRRYIRS) (40 μM) for 1 hour at 37°C. After being washed, 20 μL of cell culture (10⁶ cells/mL) was placed into wells of glass slides precoated with poly-L-lysine and incubated for 15 minutes. Cells were then washed, and fixed with 4% paraformaldehyde in PBS (pH 7.4). Cells were permeabilized and blocked with 20 μL of 5% human AB serum, 5% goat serum, 0.1% NP-40, 0.01% saponin (Calbiochem-Novabiochem Corp.), and 1% milk in PBS. They were then washed, and incubated with anti-IRS mAb (IgG1; Covance Research Products Inc., Rich-

Figure 1
A simplified model depicting the pathways leading from the IL-2R to cell cycle progression (adapted from ref. 17). Pathways that may be indirect are shown with dashed arrows. PI-3K, PI 3-kinase; Rb, retinoblastoma protein; PKC, protein kinase C; FKBP, PK-506 binding protein.
mond, California, USA) and anti-HLA class I mAb (W6/32, IgG2a) for 1 hour at room temperature. After cells were washed, 20 μL of secondary antibodies (biotin-conjugated anti-mouse IgG, and FITC-conjugated anti-mouse IgG2a from PharMingen) were added, followed by streptavidin Alexa Fluor 594 conjugate and anti-fluorescein Alexa Fluor 488 conjugate (Molecular Probes Inc., Eugene, Oregon, USA). The slides were analyzed on a MultiProbe 2010 laser scanning confocal microscope from Molecular Dynamics (Sunnyvale, California, USA).

Results

**DQ 65-79 does not inhibit expression or phosphorylation of the IL-2R.** We previously demonstrated that the DQ 65-79 peptide inhibited IL-2-mediated cell proliferation (15). The high-affinity IL-2R is composed of the α, β, and γ chains, which are upregulated to various degrees upon stimulation (21). Treatment of activated peripheral blood leukocytes with the peptide or rapamycin had no effect on the expression of any of these chains, as determined by flow cytometry, when compared with the DMSO media control or DQ 65-79(72D) control peptide (not shown). In addition, tyrosine phosphorylation (22, 23) of the β and γ chains was not affected by DQ 65-79, DQ 65-79(72D), or rapamycin (data not shown). Thus, the anti-proliferative effect of the peptide occurs downstream of IL-2R phosphorylation.

**DQ 65-79 has no effect on general tyrosine phosphorylation or on nonreceptor tyrosine kinase activity.** Although no effect of DQ 65-79 on IL-2R phosphorylation was detected, it was possible that other proximal events were affected by the peptide. Tyrosine phosphorylation of proteins is one of the earliest events to occur after activation (24). Phosphotyrosine proteins were immunoprecipitated from IL-2–stimulated T cells, and probed with anti-phosphotyrosine Ab. No change in the pattern of phosphotyrosine proteins was observed with DQ 65-79 on IL-2–stimulated cells compared with controls (Figure 2a).

The Jak kinases, Jak1 and Jak3, are key mediators of IL-2R signal transduction. In particular, Jak3, which is restricted in expression primarily to activated leukocytes, is critical for signaling, as demonstrated by the Jak3−/− mouse (25, 26). Jak1 associates with the IL-2R β chain, and Jak3 associates with the IL-2R γ chain; both become tyrosine phosphorylated after activation of the receptor (27). No change in the phosphorylation of Jak1 or the association of Jak3 with the IL-2R γ chain were observed in cells treated with peptide (data not shown).

The Src kinase family members Lck and Fyn associate with the IL-2R β chain (28, 29), and become phosphorylated upon activation of the receptor (30). Phosphorylation of these kinases can lead to the recruitment of other proteins involved in signaling, including PI 3-kinase (31, 32). Peptide treatment did not alter the amount of tyrosine-phosphorylated Fyn or Lck immunoprecipitated from activated cells (Figure 2b), or their kinase activity (Figure 2c). Therefore, treatment with DQ 65-79 does not inhibit phosphotyrosine activity in general, nor does it affect the activity of several proximal tyrosine kinases.

**DQ 65-79 blocks p70 S6 kinase activity.** We demonstrated that DQ 65-79 blocks events that are critical for cell-cycle progression, with the most proximal event identified being p27 downregulation (15). Rapamycin also inhibits degradation of p27 (14) and other distal signaling molecules, including p70 S6 kinase (33, 34). This kinase is important, but not essential, for the induction of the proliferative response; cells lacking the p70 S6 kinase gene still proliferate, but at a slower rate (35). This kinase phosphorylates the ribosomal protein S6, leading to an increase in translation of ribosomal and elongation factor mRNAs (36). Rapamycin inhibits p70 S6 kinase activity indirectly by blocking the function of upstream mTOR, which directly phosphorylates p70 S6 kinase (37). Cells treated with DQ 65-79 or rapamycin exhibit impaired p70 S6 kinase activity (Figure 3). Peptide or rapamycin added directly to the

Figure 2

DQ 65-79 does not inhibit phosphotyrosine activity upon IL-2 stimulation. (a) Western blot using anti-phosphotyrosine mAb. (b) Western blot using anti-Fyn or anti-Lck. (c) Kinase activity of immunoprecipitated Fyn and Lck. NS, no stimulation; D, DMSO; Q, DQ 65-79; 72, DQ 65-79(72D); R, rapamycin.
kinase assay had no effect (data not shown). Thus, another distal event in IL-2 receptor signaling is blocked by both DQ 65-79 and rapamycin.

Unlike rapamycin, DQ 65-79 inhibits Akt kinase activity. Akt, also known as protein kinase B, is a serine/threonine kinase in the IL-2R pathway that is not inhibited by rapamycin. Akt is involved in proliferation (38, 39), prevention of apoptosis (39, 40), and insulin signal transduction (41–43). The fungal metabolite wortmannin acts upstream of Akt, covalently binding to the p110 catalytic subunit of PI 3-kinase, blocking its kinase activity (44). Akt kinase activity was inhibited by treatment of cells with either DQ 65-79 or wortmannin, but not by rapamycin (Figure 4). This effect was indirect – peptide added to the kinase assay had no effect (data not shown). Thus, DQ 65-79 differs from rapamycin by blocking IL-2R signaling proximal to Akt.

DQ 65-79 blocks PI 3-kinase activity in vitro. Intriguingly, residues 70–79 of DQ 65-79 are highly homologous to a region of the catalytic subunit of PI 3-kinase (Table 1), prompting us to examine the effect of DQ 65-79 on PI 3-kinase activity. PI 3-kinase is composed of a regulatory and a catalytic subunit, and there are several isoforms of each subunit (45). The regulatory subunit (p85) associates with the IL-2R upon stimulation, either directly or indirectly, by binding to phosphorylated tyrosine residues (46). PI 3-kinase has been shown to be directly associated with the IL-2R β chain (46), Fyn (31), Lck (32), IRS (47), and Grb2 (48). This association brings the catalytic (p110) subunit proximal to its lipid substrates (49). DQ 65-79 did not affect either subunit association or the interaction of the regulatory subunit with phosphorylated proteins (Figure 5).

Because DQ 65-79 did not alter the physical interactions of PI 3-kinase, we next tested the effects of the peptide on PI 3-kinase activity. A lipid kinase assay was performed with PI 3-kinase immunoprecipitated from peptide-treated cells. Whereas wortmannin blocked PI 3-kinase activity, no effect was detected with DQ 65-79 (Figure 6a). However, the effect of the peptide might not be apparent if the peptide did not bind directly to the kinase. The region of homology with the p110 subunit is in the catalytic part of the molecule rather than the region of subunit association (50), and the peptide has no effect on the association of the subunits. PI 3-kinase activity was blocked when DQ 65-79 was added directly to the kinase assay (Figure 6b). Therefore, DQ 65-79 blocks the catalytic activity of PI 3-kinase in vitro in a manner distinct from wortmannin.

Table 1
DQ 65-79 is homologous to the p110 subunit of PI-3 kinase

<table>
<thead>
<tr>
<th>Sequence alignment</th>
<th>Identity (%)</th>
<th>Homology (%)</th>
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<tbody>
<tr>
<td>DQ 65-79</td>
<td>K H N L N I V I K R</td>
<td>50 80</td>
</tr>
<tr>
<td>PI-3K p110-α</td>
<td>R H N S N I M V K D</td>
<td>50 80</td>
</tr>
<tr>
<td>PI-3K p110-β</td>
<td>R H S D N I M V K K</td>
<td>40 80</td>
</tr>
<tr>
<td>PI-3K p110-γ</td>
<td>R H N D N I M H T E</td>
<td>50 70</td>
</tr>
<tr>
<td>PI-3K p110-δ</td>
<td>R H S D N I M H E</td>
<td>40 70</td>
</tr>
<tr>
<td>PI-4K p110</td>
<td>R H N G N I M L D K</td>
<td>40 80</td>
</tr>
<tr>
<td>mTOR</td>
<td>R H P S N L M L D R</td>
<td>30 70</td>
</tr>
</tbody>
</table>

Identical residues are in bold and similar residues are underlined. PI-3K, PI-3-kinase.
Our initial hypothesis was that DQ 65-79 physically interfered with the interaction between T cells and antigen-presenting cells by binding to the T-cell receptor. However, our findings do not support this. DQ 65-79 inhibits not only allorecognition but also proliferation of purified T cells in response to anti-CD3 or IL-2, indicating that antigen-presenting cells are not required for inhibition. Interestingly, the peptide does not inhibit PMA- and ionomycin-mediated proliferation (15), implying a membrane-associated effect. Additionally, DQ 65-79 inhibits intracellular signaling events in a manner similar to but distinct from the immunosuppressive drugs rapamycin and wortmannin, suggesting that DQ 65-79 acts within the cell. The observation that DQ 65-79 is rapidly internalized in cells (Drouvalakis and Krensky, manuscript in preparation) supports an intracellular locus for peptide action. Thus, the functional activities and physical location of DQ 65-79 are consistent with inhibition of PI 3-kinase activity.

Although the mechanism by which DQ 65-79 crosses the membrane has not been established, other peptides also translocate into cells. For example, conjugation of proteins to short cationic peptides enhances transport of proteins into cells (51, 52). Zhang and coworkers prepared cell-permeable synthetic peptides by incorporation of a variety of hydrophobic sequences (53). DQ 65-79 is mainly composed of hydrophobic and positively charged residues. Substitution analysis showed that replacement with serine at all but two of these residues resulted in a reduction of inhibitory activity (15). Thus, it is possible that the ability of DQ 65-79 to translocate into the cytosol is related to its overall hydrophobicity and positively charged amino acids.

Murphy and coworkers recently described a peptide designated HLA-DQAI that inhibits allorecognition by inducing apoptosis (54). This peptide is derived from residues 62–77 of the α chain of DQA*0101, but differs
from DQ 65-79 in several ways: (a) HLA-DQA1 is three residues longer than DQ 65-79 at the amino terminus; (b) HLA-DQA1 is two residues shorter than DQ 65-79 at the carboxyl terminus; and (c) the core region of the two peptides differs at residues 66, 69, and 76. It will be interesting to determine whether these two related peptides have similar mechanisms of action.

We have not yet determined exactly how DQ 65-79 interferes with PI 3-kinase activity. The region of homology between the peptide and p110 is within the catalytic region of the subunit that contains several functional subdomains (50). The DQ 65-79 homology region does not correspond to the ATP-binding region (amino acids 802–825) (50), to the covalent attachment site of wortmannin (amino acid 802) (55), or to the putative head binding region (amino acids 937–952) (56); rather, it corresponds to part of the kinase domain (amino acids 916–924) (50). Interestingly, a change at position 916 of p110-β from arginine to lysine causes a complete loss of kinase function (57). The corresponding residue in DQ 65-79 is lysine instead of arginine, which may account for the inhibitory effects of the peptide. However, we are unable to demonstrate direct interaction between DQ 65-79 and PI 3-kinase, ATP, or the lipid substrate (Boytim and Clayberger, unpublished observations). The interaction of the peptide with the components of the in vitro kinase assay may be too weak to be detected by conventional methods. Alternatively (though it is not likely), the peptide may be acting through an associated protein that is coimmunoprecipitated with the kinase.

Due to the ubiquitous expression of PI 3-kinase and its role in many different signaling pathways, one might expect that DQ 65-79 would inhibit multiple cell types and multiple effector functions of those cells. We have observed a range in sensitivity to the antiproliferative effects of the peptide with different cell types, with lymphocytes being more sensitive than other types of cells. This may be attributed to the levels of different isoforms of PI 3-kinase, although it is not yet clear what role the different isoforms play in signaling specificity. It is intriguing that the p110-β isoform is primarily leukocyte specific (58), which may provide an explanation for the more potent effects of DQ 65-79 on lymphocytes vs. other cell types.

DQ 65-79 may also inhibit the activity of other PI kinase family members. Wortmannin has been shown to block the kinase activity of PI 4-kinase (59), mTOR (60), and Vps34p (61). The PI kinase family member mTOR also has homology to DQ 65-79 in its kinase domain. Although no lipid kinase activity has been demonstrated for mTOR (56), the peptide may affect other activities of mTOR. Inhibition of multiple members of the IL-2R signaling pathway may explain the strong inhibitory effect of the peptide on proliferation, but the effect of the peptide on mTOR and other PI kinase family members remains to be investigated. It is also possible that the peptide may interact with other proteins within the cell. Therefore, although we have demonstrated the inhibition of PI 3-kinase as a primary action of DQ 65-79, other effects may also be involved in the inhibition of proliferation.

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