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Proliferating Cell Nuclear Antigen as the Cell Cycle Sensor for an HLA-Derived Peptide Blocking T Cell Proliferation

Xuefeng Ling,* Salar Kamangar,* Michelle L. Boytim,† Zvi Kelman,‡ Philip Huie,§ Shu-Chen Lyu,† Richard K. Sibley,§ Jerard Hurwitz,‡ Carol Clayberger,*† and Alan M. Krensky2*†

Synthetic peptides corresponding to structural regions of HLA molecules are novel immunosuppressive agents. A peptide corresponding to residues 65–79 of the α-chain of HLA-DQA03011 (DQ65–79) blocks cell cycle progression from early G1 to the G1 restriction point, which inhibits cyclin-dependent kinase-2 activity and phosphorylation of the retinoblastoma protein. A yeast two-hybrid screen identified proliferating cell nuclear Ag (PCNA) as a cellular ligand for this peptide, whose interaction with PCNA was further confirmed by in vitro biochemistry. Electron microscopy demonstrates that the DQ65–79 peptide enters the cell and colocalizes with PCNA in the T cell nucleus in vivo. Binding of the DQ65–79 peptide to PCNA did not block polymerase δ (pol δ)-dependent DNA replication in vitro. These findings support a key role for PCNA as a sensor of cell cycle progression and reveal an unanticipated function for conserved regions of HLA molecules. The Journal of Immunology, 2000, 164: 6188–6192.

Materials and Methods

Yeast two-hybrid screen

The yeast two-hybrid screen was performed as described in the MATCHMAKER Two-Hybrid System (Clontech, Palo Alto, CA). Synthetic oligonucleotides encoding residues 65–79 of HLA-DQA03011 were designed using the most frequent codon for each amino acid. The peptide sequences were fused to the carboxyl terminus of the GAL4 DNA-binding domain in plasmid vector pAS1 (a gift from Dr. S. J. Elledge, Baylor College of Medicine), which results in the plasmid pAS-DQ (also named pXL-17). pAS-B2702, which has HLA class I B2702 84–75/75–84 peptide sequences, was constructed similarly as pAS-DQ. Other control plasmids pAS-lamin, pAS-p53, and pAS-CDK2 are gifts from Dr. S. J. Elledge. The plasmid vector, pACT2, was purchased from Clontech. Poly-A mRNA was extracted from human PBL 6 days after PHA-P activation and was reverse transcribed into cDNA. The cDNA expression library was generated by fusing cDNA to the carboxyl terminal of the GAL4 activation domain in the pACT2 plasmid, yielding a complexity >106. The library and pAS-DQ plasmids were cotransformed into the yeast strain Y190 (from Clontech), and screens were performed as described by Clontech matchmaker yeast two-hybrid protocol. “Interacting” clones isolated in the library screen were retransformed with either with pAS-DQ or with other “bait” plasmids. Cotransformation experiments were performed in duplicate, and transformants were plated with synthetic minimal media SD-Trp-Leu-HIS plus 3AT (30 mM 3-amino-1,2,4-triazole, catalogue no. A-8056, Sigma, St. Louis, MO). After incubation at 30°C for 3 days, a β-galactosidase filter assay was performed. Clones positive with pAS-DQ but not with other “bait” plasmids were further characterized. Library plasmids were isolated from positive clones and sequenced (Sequenase 1.0 DNA Sequencing Kit, United States Biochemical, Cleveland, OH). The DNA sequences generated were analyzed for homology in the GenBank database (http://www.ncbi.nlm.nih.gov).

In vitro binding of PCNA to peptide

Peptides were synthesized and HPLC purified as described (3). Where indicated, a six-histidine-tag (HIS-) was added to the amino terminus. The histidine tag did not affect the anti-proliferative function of DQ65–79 peptide (data not shown). Stock peptide solutions were prepared in DMSO, and exact concentrations were determined by amino acid analysis (PAN Systems, Waltham, MA). After incubation at 30°C for 3 days, Peptide/PCNA interaction in vitro was assayed by ELISA. Microtiter wells were coated with 200 μl of 1 μM recombinant purified PCNA in PBS overnight at 4°C, washed with PBST (PBS plus 0.1% Tween 20), then blocked with 5% nonfat milk in PBST for
2 h at room temperature. Wells were then incubated with 1) peptide for 1 h at room temperature; 2) anti-tetra histidine mAb (Qiagen, Chatsworth, CA) diluted to 1:1000 in PBST for 1 h at room temperature; 3) HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and goat anti-rabbit-10 nm colloidal gold (AutoProbe EM GAR G10, Amersham Life Sciences, Arlington Heights, IL). His-DQ65–79 was labeled by anti-penta HIS (BSA free, mouse IgG1 mAb, Qiagen) and goat anti-mouse IgG-30 nm colloidal gold (AutoProbe EM GAR G30, Amersham Life Sciences). PCNA and His-DQ65–79 were applied in two separate rounds of staining. Briefly, each round consisted of the application of a primary Ab blocking buffer to the sections for overnight incubation at 4°C, then incubation with the appropriate secondary Ab in blocking buffer and/or colloidal gold solution, each for 3 h at room temperature. The sections were then washed in distilled water and lightly counterstained with uranyl acetate and lead citrate. The sections were viewed with a Hitachi H-300 electron microscope (Hitachi, Tokyo, Japan).

Electron microscopy

PBL were isolated by Ficoll-Hypaque density centrifugation and cultured for 6 days in RPMI 1640 supplemented with 10% FCS (HyClone, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μg/ml PHA-P. On day 6, 5 × 10^6 PBL were incubated with either 40 μM HIS-DQ or the equivalent amount of DMSO for 1 h and then fixed with 5% glutaraldehyde in PBS (Sigma) for 1 h at room temperature. The cells were washed in several changes of PBS, dehydrated in a series of ethanol treatments, and embedded in LR White (Electron Microscopy Sciences, Fort Washington, PA). Thin sections were blocked in PBS containing 5% BSA and 0.5% goat serum for 1 h at room temperature. PCNA was labeled by sheep anti-human PCNA (The Binding Site, Birmingham, U.K.), rabbit anti-sheep IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), and goat anti-rabbit-10 nm colloidal gold (AutoProbe EM GAR G10, Amersham Life Sciences, Arlington Heights, IL). His-DQ65–79 was labeled by anti-penta HIS (BSA free, mouse IgG1 mAb, Qiagen) and goat anti-mouse IgG-30 nm colloidal gold (AutoProbe EM GAR G30, Amersham Life Sciences). PCNA and His-DQ65–79 were applied in two separate rounds of staining. Briefly, each round consisted of the application of a primary Ab blocking buffer to the sections for overnight incubation at 4°C, then incubation with the appropriate secondary Ab in blocking buffer and/or colloidal gold solution, each for 3 h at room temperature. The sections were then washed in distilled water and lightly counterstained with uranyl acetate and lead citrate. The sections were viewed with a Hitachi H-300 electron microscope (Hitachi, Tokyo, Japan).

DQ65–79 peptide enters the cell and colocalizes with PCNA in the T cell nucleus in vivo

Characterization of the interaction of PCNA and the DQ65–79 in vitro suggests that the anti-proliferative effect of the DQ65–79 peptide may be due to its binding of PCNA in vivo. If the binding of the DQ65–79 peptide to PCNA blocks cell cycle progression, the DQ65–79 peptide should enter the cell and colocalize with PCNA. To test this hypothesis, electron microscopy experiments were performed. As shown in Fig. 2, in His-DQ65–79 peptide-treated T cells, His-DQ65–79 (labeled by 30 nm gold particles) was found almost exclusively in the nucleus and in very close proximity to PCNA (labeled by 10-nm gold particles) in numerous instances per grid. When both primary Abs were omitted, no gold particles were detected, demonstrating that the secondary Abs specifically recognize the primary Abs rather than nonspecific epitopes in other proteins. In DMSO-treated T cells, PCNA distribution was essentially identical with that of the samples treated with the His-DQ65–79 peptide. These findings clearly demonstrate that DQ65–79 crosses the cell plasma membrane and interacts physically with PCNA in vivo in the T cell nucleus.

**Table 1. Clones identified by the yeast two-hybrid screen**

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of Independent Clones</th>
<th>Specific Interaction with DQ65–79*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFP35</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>PCNA</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>Actin-binding protein</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>Ras-related protein</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td>NKTR</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Cyclin G</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>Unknown’a</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>Unknown’b</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>A Zap-like sequence’c</td>
<td>1</td>
<td>ND</td>
</tr>
<tr>
<td>DNA polymerase’d</td>
<td>1</td>
<td>–</td>
</tr>
</tbody>
</table>

* Positive in β-galactosidase colony-lift assay and His 3 expression as judged by growth on plates containing 3-aminotriazole.

’a Last 12 residues of carboxyl terminus.

*b Very short open reading frame.

c Homology to Leishmania donovani.

d Homology to Leishmania major.

The DQ65–79 peptide does not block pol δ-dependent DNA replication in vitro

PCNA plays important roles in DNA replication, DNA repair, and regulation of the cell cycle. PCNA interacts with DNA pol δ and replication factor C to form a processive DNA polymerase complex (9–11). Synthetic peptides corresponding to p21, a cell cycle inhibitor, bind PCNA and block the function of PCNA in the DNA polymerase complex in vitro (12). To determine whether DQ65–79 affects DNA replication by direct binding to PCNA, peptides were demonstrating that the DQ65–79/PCNA interaction was direct and did not require intermediate or additional factors. To define the residues within the DQ65–79 peptide critical for PCNA binding, a panel of synthetic peptides was prepared in which a six-histidine tag was added to the amino terminus for detection and each residue was individually substituted with serine (Fig. 1C). Substitution at a cluster of three hydrophobic residues (isoleucine at position 75, valine at 76, and isoleucine at 77) prevented the binding of the DQ65–79 peptide to PCNA. With the exception of the His-73S peptide, there was a strong correlation between the binding of each peptide to PCNA and the ability of that peptide to inhibit T cell proliferation.

Electron microscopy demonstrates that the DQ65–79 peptide affects DNA replication by direct binding to PCNA, peptides were...
added to an in vitro system that measures pol δ-dependent DNA synthesis (7). None of the peptides tested, DQ65–79, DQ74S, and DQ72D, had an effect on pol δ-dependent DNA synthesis (Fig. 3), demonstrating that the anti-proliferative effect of DQ65–79 does not involve effects on DNA pol δ replication per se.

The DQ65–79 peptide inhibits the phosphorylation of Rb protein

To evaluate the effects of the DQ65–79 peptide on the G1 check protein Rb, activated T cells were treated either with the inhibitory DQ65–79 peptide or with a peptide in which glutamine was substituted for aspartic acid at residue 72 (DQ72D). This substituted peptide does not inhibit T cell proliferation (6) and does not bind to PCNA in vitro (data not shown). Rb was normally phosphorylated in cells incubated with DQ72D, but was not phosphorylated in DQ65–79-treated cells (Fig. 4). Both rapamycin and DQ65–79 block cell cycle progression in a similar fashion (6), and we found that Rb was hypophosphorylated in cells treated with either DQ65–79 or rapamycin (Fig. 4), underscoring the mechanistic similarity between these two immunosuppressive agents. The Rb phosphorylation pattern also suggests that DQ65–79 and rapamycin both affect some cell cycle control point upstream of the Rb in the early G1 to G1 restriction point regulation pathway.

Discussion

We have previously shown that synthetic peptides corresponding to conserved HLA regions have immunosuppressive functions (1–6). In this report, we investigated the mechanism by which DQ65–79 blocks T cell proliferation and cell cycle progression. A yeast two-hybrid screen identified PCNA as the cellular ligand for the DQ65–79 peptide, and this interaction was further confirmed by in vitro biochemistry and electron microscopy.

Synthetic peptides derived from p21 interact with PCNA to block replication in vitro but have no effect on DNA synthesis in

FIGURE 1. The interaction of DQ65–79 and its serine-substituted analogues with PCNA in vitro correlates with the anti-proliferative function of the peptides. A, Concentration-dependent inhibition of T cell proliferation by HIS-peptides. Inhibition of [3H]TdR incorporation by the CD8+ T cell line AJY in the presence of the indicated concentration of either HIS-DQ65–79 (▲) or HIS-DQ65–79 substituted with serine at residue 75 (HIS-75S; ●). B, Concentration-dependent binding of HIS-peptides to PCNA in vitro. Binding of HIS-DQ65–79 (▲) or HIS-75S (●) to microtiter wells coated with recombinant PCNA was measured by ELISA. In the absence of PCNA, neither HIS-DQ65–79 nor HIS-75S gave any measurable binding above background (data not shown). C, Correlation of HIS-DQ65–79 and HIS-tagged serine mutant peptides binding to recombinant PCNA and inhibition of T cell proliferation. The final peptide concentration is 40 μM for both wild-type (NIAVLKHNLNIVIKR) and substituted peptides. The residue substituted with serine is indicated by the number beneath each bar on the graph. Percentage binding to PCNA was calculated as [(OD HIS-serine substituted peptide)/(OD HIS-DQ65–79 peptide)] × 100. Percentage inhibition of proliferation was calculated as [(cpm (no peptide) − cpm (peptide))/(cpm (no peptide) − cpm (40 μM DQ65–79 peptide))] × 100.

FIGURE 2. The DQ65–79 peptide enters the T cell and colocalizes with PCNA in the nucleus. PCNA is labeled with 10-nm gold particles, and the DQ65–79 peptide is labeled with 30-nm gold particles.
We propose that the DQ65–79 peptide interacts with PCNA in the CDK/cyclin/p21/PCNA quaternary complex, sending negative signals that affect the early G₁ to G₁ restriction point transition. This proposed model is based largely on the following observations: PCNA is a component of the CDK/cyclin/p21/PCNA quaternary complex (13–15); DQ65–79 interacts with PCNA in activated T cell nuclei, and its anti-proliferative effects strongly correlate with the strength of this interaction; DQ65–79 blocks the early G₁ to G₁ restriction point transition but does not block DNA replication. With cellular transformation, the subunits rearrange in the CDK/cyclin/p21/PCNA quaternary complex (14) with an accompanying loss of cell cycle regulation. Our result demonstrates that the DQ65–79 peptide blocks early G₁ to G₁ restriction point transition as Rb is hypophosphorylated in activated T cells. Collectively, these findings support our model that the DQ65–79 peptide interacts with PCNA and negatively affects the “sensor” function of PCNA operative at the early G₁ to G₁ restriction point transition.

Rapamycin is a potent anti-proliferative macrolide that has recently been approved for use in clinical transplantation (20). Both rapamycin and DQ65–79 block cell cycle progression at the early G₁ to G₁ restriction point interface and act downstream of the IL-2 receptor (6). Rb phosphorylation is blocked in T cells treated with rapamycin in a similar fashion as DQ65–79 (Fig. 4). Interestingly, rapamycin down-regulates PCNA at the transcriptional level (21). Most likely, both rapamycin and DQ65–79 negatively affect the “sensor” function of PCNA at early G₁ to G₁ restriction point transition: rapamycin decreases the amount of PCNA while DQ65–79 exerts its negative influence through protein-protein interactions.

Peptides derived from HLA molecules modulate immune response both in vitro and in vivo, suggesting that conserved regions of HLA may have important cellular functions. A synthetic peptide corresponding to residues 75–84 of HLA-B2702 induces T cell anergy, perhaps via interaction with members of the heat shock protein 70 family (4). The DQ65–79 peptide, derived from an HLA class II sequence, is anti-proliferative, blocking cell cycle progression at the early G₁ to G₁ restriction point transition (6). In the present study, we show that the DQ65–79 peptide interacts with PCNA in the nucleus, providing an unexpected molecular explanation for this anti-proliferative effect. The DQ/PCNA interaction may send a negative signal through the CDK/cyclin/p21/PCNA quaternary complex to block cell cycle progression at the early G₁ to G₁ restriction point transition, adding new evidence to previous proposals that PCNA functions as a cell cycle “sensor”
These findings further suggest that conserved regions of HLA molecules mediate heretofore unappreciated cellular functions. Our observation identifies PCNA as a potential target for the design of novel synthetic compounds that may prove clinically useful as anti-proliferative agents by blocking cell cycle progression.

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References