Glyceraldehyde 3-phosphate dehydrogenase is a novel autoantigen leading autoimmune responses to proliferating cell nuclear antigen multiprotein complexes in lupus patients

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Keywords: antigens, autoantibodies, human, systemic lupus erythematosus

Abstract
Using 2-dimensional electrophoresis and ion-pair chromatography, we have identified elements of proliferating cell nuclear antigen (PCNA) multiprotein complexes that are reactive to antibodies in sera from patients with systemic lupus erythematosus. Among the various elements of the complexes, a 37 kDa protein (PI 8.5) that specifically reacted with SLE sera, but not with sera from patients with other connective tissue diseases, was identified as glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Immunoblot analysis showed that SLE sera reactive with the 37 kDa protein specifically reacted with GAPDH, as did anti-GAPDH mAbs. The purified autoantibodies to GAPDH from lupus serum showed both nuclear speckled and cytoplasmic staining patterns in immunofluorescence on Hep-2 cells. In addition, enzyme-linked immunosorbent assay (ELISA) revealed the presence of anti-GAPDH autoantibodies in 47% of lupus patients. Longitudinal analysis of the reactivity of lupus sera to PCNA complexes showed the autoimmune response to spread from GAPDH to other elements of PCNA complexes, and the presence of anti-GAPDH antibodies was significantly correlated with increased levels of serum PCNA. Taken together, these findings suggest that GAPDH interacting with PCNA in association with its cellular function is a novel autoantigen recognized by lupus sera, and that GAPDH thus plays an important role in the induction of autoimmune responses against the PCNA complex.

Introduction
Proliferating cell nuclear antigen (PCNA) is known to be the autoantigen specifically recognized by antibodies in sera from patients with systemic lupus erythematosus (SLE) (1). We have determined PCNA to be a 34 kDa intranuclear poly-peptide (2,3) whose expression is increased during the late G1 to S phases of the cell cycle, immediately before DNA synthesis (4). These findings suggested a strong association between PCNA and DNA replication, which was consistent with the subsequent finding that PCNA is an auxiliary protein of DNA polymerase-α (Pol-α), which plays an essential role in DNA replication and repair (5–9). Further analysis of the structure and function of PCNA revealed it to be a toroidally shaped trimer that interacts with many proteins to form a very large protein complex called a ‘DNA replication fork’ which enables individual polymerases to efficiently synthesize long DNA strands (10–12). It is now known, however, that the proteins binding to PCNA are not limited to enzymes involved in the mechanics of DNA replication and repair (13), but also include p21, cyclin D and Gadd45, molecules associated with cell cycle regulation (14–17); human DNA-(cytosine-5) methyltransferase (MCMT), a protein associated with DNA methylation (18); CAF1, an essential factor for the coupling of chromatin assembly (19); and Ku 70/80, topoisomerase (Top) I, TFIIID, SL-1 and ribosomal DNA (rDNA) transcription terminator factor (TTF-1), all of which are associated with the pre-assembled RNA polymerase I holoenzyme complex necessary to initiate transcription of rDNA (20).

We previously showed that a group of anti-PCNA monoclonal antibodies (mAbs) raised in our laboratory can react with PCNA bound to or interacting with other proteins.
associated with cell proliferation (PCNA complexes) (21,22) and that PCNA complexes purified from rabbit thymus extract (RTE) by affinity chromatography using these anti-PCNA mAbs also react with antibodies against p21, replication protein A (RPA), DNA helicase II (NDH II), cyclin-dependent kinase (CDK)4, CDK5 and Top I (22). This suggests that a substantial part of the 'protein machinery' for DNA replication and cell cycle regulation purifies as PCNA complexes reacting with anti-PCNA mAbs.

We have also shown that 35% (14 out of 40) of SLE sera react with at least one component of the PCNA complex (23), which suggests that analysis of the targeted antigens may shed light on the mechanisms underlying autoimmune responses to proteins interacting with PCNA in SLE patients. In the present study, therefore, we purified PCNA complexes using anti-PCNA mAbs and identified the components of the PCNA complex using two-dimensional (2D)-PAGE and ion-pair chromatography. In particular, we discuss the biological function of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was identified as a PCNA binding protein, and its possible role in the induction of autoimmune responses against PCNA complexes in SLE patients.

Methods

Sera

Anti-PCNA serum AK was selected from the serum bank at Juntendo University Hospital using anti-PCNA standard serum PT (kindly provided by Dr Eng M. Tan, Autoimmune Research Center, The Scripps Research Institute, La Jolla, CA) in double immunodiffusion (DID) assays as previously described (2,4). Serum AK subsequently served as the source of the anti-PCNA IgG used to make an affinity chromatography column and was also used to monitor the antigenic activity of PCNA in DID and immunoblot (IB) assays carried out during the antigen purification. Standard sera containing autoantibodies to other nuclear antigens, including U1 RNP, Sm and Top I were also provided by Dr Tan.

The reactivity of the following sera with elements of the PCNA complex and GAPDH were evaluated: 75 sera from SLE patients, 24 from rheumatoid arthritis (RA) patients and 21 from scleroderma (SSc) patients, all of whom met the criteria for diagnosis proposed by the American College of Rheumatology (24–26); 20 from polymyositis (PM)/dermatomyositis for diagnosis proposed by the American College of Rheumatology, was coupled to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ). This was followed by affinity chromatography using 2 mg of mAb (TOB7 and TO30) per ml of Sepharose 4B gel (30). Anti-PCNA gels were then packed into a Bio-Rad econo-column (Bio-Rad, Richmond, CA) and PCNA-containing RTE was passed over the column at a rate of 5 ml/h. After washing with more than three column bed volumes of PBS and 1 M NaCl/0.01 M phosphate buffer (pH 7.4), the bound material was eluted with 3 M NaSCN, and the eluate was dialyzed against PBS.

SDS–PAGE

The protein profile of the purified PCNA was analyzed by SDS–PAGE (2). The slab gel consisted of 12.5% acrylamide and 0.1% SDS in Tris–HCl (pH 6.8) with 15 mm of stacking gel containing 5% acrylamide and 0.1% SDS in Tris–HCl (pH 6.8). Samples dissolved in sample buffer (3% SDS, 5% 2-mercaptoethanol, 55 mM Tris HCl, pH 6.8, 10% glycerol, bromphenol blue) and boiled for 3 min were electrophoresed (1.5 mA/cm) at 4°C, and the resultant bands were stained with Coomassie Blue R250.

IB assays

Purified PCNAs were transferred electrophoretically onto nitrocellulose filters (Bio-Rad) as previously described (2,30). For immunochromic detection of proteins, the filters were first blocked for 24 h with 3% BSA in 0.1% Tween–PBS (T-PBS) and then incubated for 2 h with anti-PCNA mAb in T-PBS (2.5 μg/ml) or with sera from patients (anti-PCNA serum AK, anti-Sm or U1 RNP serum YT, or anti-Top I serum YM; 1:200 dilution in T-PBS). After washing with T-PBS, the filters were incubated for 2 h with HRP-conjugated goat anti-mouse γ-globulin (1:1000 dilution in T-PBS; Cappel, West Chester, PA) or HRP-conjugated goat anti-human IgG (1:2000 dilution; Cappel). After a final wash, the bound conjugate was detected by incubation with substrate solution (250 μg of 3,3-diaminobenzidine-4 HCl/m, 0.5 μl of 30% H2O2/ml, 0.05 M Tris–HCl buffer, pH 7.6) and the resultant bands were stained with 0.1% amido black 10B in 7% acetic acid.

These mAbs were also used as probes for the PCNA complex in immunoblot assays.

mAbs against molecules associated with cell proliferation and GAPDH

IB assays using murine mAbs against various molecules associated with cell proliferation were used to investigate the components of the PCNA complex. Used were mAbs against human p21 (Biosource, Camarillo, CA), RPA (NeoMarkers, Fremont, CA), NDH II (Cosmo Bio, Tokyo, Japan), CDK4 (NeoMarkers) and CDK5 (NeoMarkers). A murine mAb against GAPDH (Chemicon International, Inc., Temecula, CA) was also used to identify GAPDH in immunoblot assays. Each of the respective mAbs were used according to the manufacturers' instructions.
Antibodies against GAPDH were detected in the same manner using rabbit GAPDH (Sigma Chemical Co., St Louis, MO) as an antigen source. Sample serum was diluted to 1:200 and mAbs to GAPDH (Chemicon) were diluted to 10 μg/ml.

Purification of anti-GAPDH antibodies and immunofluorescent staining

In order to analyze the cellular localization of GAPDH, anti-GAPDH antibodies in sera from lupus patient IS were purified using nitrocellulose membranes onto which GAPDH had been transferred. The membranes were cut into small pieces and blocked overnight in T-PBS/milk, after which the pieces of membrane were incubated for 2 h in serum IS diluted 1:50. The membranes were then washed twice for 10 min each in T-PBS and the pieces were incubated for 5 min at room temperature with 200 mM KPO4/150 mM NaCl (pH 2.5) to elute the autoantibodies against GAPDH, followed by neutralization with 1 M Tris buffer (pH 8.7). This purification procedure was repeated several times, after which the eluted autoantibodies were concentrated using Microcon 30 microconcentrators (Amicon, Inc. Beverly, MA). For immunofluorescent staining, the purified autoantibodies and serum IS (diluted to 1:100) were incubated on Hep-2 cell smears (MBL, Nagaya, Japan) and reactions were detected using FITC-labeled anti-human goat IgG antibody (KPL).

2D-PAGE

The components of the purified PCNA complexes were analyzed using 2D-PAGE (31). For the first-dimensional electric focusing of the PCNA complexes, IPGphor strips (11 cm, pH 3-10; Amersham Pharmacia Biotech, Uppsala, Sweden) were used. The purified complexes were diluted with the electric focusing solution containing 9 M urea, 4% CHAPS, 65 mM dithioerythritol (DTE), 2% carrier ampholyte at pH 3-10 and bromphenol blue (BPB); dried IPG strips were rehydrated in sample solution overnight. Before the second-dimensional SDS–PAGE, the strips were immersed in solution containing 50 mM Tris–HCl, pH 8.5, 6 M urea, 30% glycerol, 2% SDS, 130 mM DTE and 0.005% BPB for 10 min in order to reduce the SH-residues of proteins, and then placed onto the SDS–PAGE gels. SDS-gel [stacking gel, 4% acrylamide, 2.6% piperazine diacrylamide (PDA) (Bio-Rad) running gel 10% acrylamide, 2.6% PDA; size 17×20×0.1 cm; Nihon Eido Co., Tokyo, Japan] electrophoresis entailed a prerun at 12 mA for 1 h, followed by a run at 24 mA for 3 h. The separated proteins on the gels were stained using a Plus One silver stain kit and 1 h, followed by a run at 24 mA for 3 h. The separated proteins were transferred electrophoretically onto nitrocellulose filters (Bio-Rad), and their reactivities were tested using various mAbs as described above. In addition, the proteins were transferred electrophoretically onto polyvinylidene fluoride (PVDF) membranes (Immobilon, pore size 0.45 μm; Millipore, Billerica, MA) using blotter (semidyry type; Nihon Eido Co.), where the amino acid composition was analyzed as previously described (31).

Ion-pair chromatography and identification of the elements of PCNA complexes

Highly sensitive 6-aminquinolyl-carbamyl (ACQ) amino acid analysis using ion-pair chromatography was carried out as previously described to identify the proteins within the PCNA complex (31,32). The proteins on PVDF membranes to which purified PCNA complexes had been transferred were cut out with a knife and then cut into two or three pieces. The pieces were then washed with 200 μl of 50% MeCN in a mixer, after which the washing solution was immediately aspirated, and the membranes were washed with 200 μl of 0.01 N HCl and the solution was again aspirated. This washing step was repeated three times, after which the membrane pieces were dried in a vacuum desiccator for 10 min and mixed with 6 N HCl (containing 2.5% phenol) in hydrolysis vessels; hydrolysis was carried out at 110°C overnight. After completely drying the membranes, they were moistened with 10 μl of neat MeCN, followed by 10 μl of 0.01 N HCl and 10 μl of fresh Milli-Q water. AQC-derivatization of the standard amino acid mixture was done by adding 10 μl of standard solution in 0.01 N HCl instead of 0.01 N HCl for the membrane sample. After, 50 μl of 0.2 M borate buffer and 20 μl of 10 mM AHC reagent were added to the samples, and the solution was left to stand for 1 min, then heated at 50°C for 10 min. Ten-microliter samples were then diluted 10-fold with 0.2 M borate buffer, after which the AQC-amino acids were separated by ion-pair chromatography using HPLC as previously described (32). The proteins were identified based on mol. wt, PI and analysis of amino acid composition using AACmplIdent tool on the ExPASy molecular biology server (32).

ELISAs

ELISAs were carried out to detect antibodies against GAPDH in lupus patients (30). Fifty microliters of rabbit GAPDH (Sigma Chemical Co.) solution (5 μg/ml) were added to the wells of Immunoplate II plates (Nunc) and incubated overnight at 4°C. These coating solutions were then removed and the plates were washed three times with T-PBS, after which 250 μl of 1% BSA in T-PBS were added to the wells and incubated for 2 h at room temperature. After washing again, 100 μl of alkaline phosphatase-labeled goat anti-human IgG (1:1000 dilution in T-PBS; KPL, Gaithersburg, MD) was added for detection of autoantibodies against GAPDH. After incubating the antibodies for 2 h at room temperature, the plates were washed and 250 μl of enzyme substrate (1 mg p-nitrophenylphosphate/ml in diethanolamine buffer, pH 9.8) was added, and the optical density (OD) at 405 nm was measured. The upper limit of the normal range was estimated based on the mean value plus 2 SD of the normal control group.

A sELISA using TOB7 and TO17 was used to measure PCNA in sera obtained from patients with various connective tissue diseases (see Sera section above) and normal subjects as previously described (21,33–35). Fifty microliters of TO17 (20 μg/ml) in carbonate buffer (pH 8.5) was used to coat wells of Immunoplate II plates (Nunc), and PCNA in 100-μl serum samples were detected by biotinylated TOB7 (5 μg/ml) and HRP-conjugated streptavidin (1:2000 dilution; BRL, Bethesda, MD). The concentration of PCNA in serum samples was calculated based on a standard PCNA curve estimated by the reaction of PCNA purified by anti-PCNA affinity chromatography using serum AK (0.002; 6 μg/ml ) as previously described (21,33–35).
Results

Purification of PCNA and IB analysis using mAbs against proteins associated with cell proliferation

After purifying PCNA complexes using anti-PCNA affinity chromatography, the polypeptide components of the purified complexes were analyzed by SDS–PAGE (Fig. 1A). Purification using serum AK yielded only a single 34 kDa band (Fig. 1A, lane 1), but purification using TO30 (lane 2) or TOB7 (lane 3) yielded a number of additional bands, as did TO17 (data not shown). IB assays carried out to evaluate the specificity of the mAbs against the 34 kDa PCNA polypeptide showed TO30 (Fig. 1B, lane 3) and TOB7 (Fig. 1C, lane 3) to react only with the 34 kDa PCNA polypeptide within the PCNA complexes, as did anti-PCNA serum AK (Fig. 1B and C, lane 2). By contrast, neither of the two negative controls (anti-U1 RNP and anti-Sm antibodies in serum TY) reacted with any of the purified polypeptides (Fig. 1B and C, lane 1). Moreover, when the elements of TOB7–PCNA were further analyzed by IB with mAbs against proteins associating with cell proliferation, p21 (Fig. 1C; lane 4), RPA (lane 5), NDH II (lane 6), Top I (lane 7), CDK4 (lane 8) and CDK5 (lane 9) were all detected, which confirmed that, as previously reported (21), multiprotein complexes associated with DNA replication and cell cycle regulation copurify with PCNA.

Analysis of the elements of PCNA complexes

Because IB analysis using various connective tissue diseases revealed that lupus sera specifically reacted with some proteins within the PCNA complexes as shown in the previous studies (22,23) (data not shown), we further analyzed the components of the complex using 2D-PAGE to improve our understanding of the autoimmune response in SLE patients, as well as such cellular functions as DNA replication and cell cycle regulation.

PCNA complexes purified from RTE (TOB7–PCNA) were separated by 2D-PAGE, transferred to a PVDF membrane and visualized by silver staining (Fig. 2). Although many proteins were present on the membrane, we focused on the amino acid composition of two: one with a PI of 4.8 and a mol. wt of 34 kDa, which seemed to be PCNA (spot A) and another with a PI of 9.13 and a mol. wt of 37 kDa, which was the largest spot on the membrane (spot B) and specifically reacted with lupus sera in the IB analysis (data not shown).

After cutting out the spots as described in the Methods, the amino acid composition was analyzed by ion-pair chromatography. Figure 3(A) shows the chromatography profile, while Fig. 3(B) shows the amino acid composition of the unknown protein in spot B deduced from the results of the chromatography. When we attempted to identify the protein using ExPASy via World Wide Web servers, we found it to be identical to GAPDH (mol. wt, 37 kDa; PI 8.5), with a rank of 1 and a score of 5. In the same way, spot A was identified as PCNA (rank, 1; score, 4; data not shown). The identity of the putative GAPDH protein was confirmed by transferring proteins separated by 2D-PAGE onto nitrocellulose paper and immunoblotting with mouse anti-GAPDH mAb. As shown in Fig. 4, the protein from spot B specifically reacted with anti-GAPDH mAb.

Reactivity of SLE sera to GAPDH

The reactivity of SLE sera with PCNA complexes by IB analysis revealed that 15 sera clearly and specifically reacted with
the 37 kDa polypeptide in TOB7–PCNA, and 10 out 15 sera reacted with purified rabbit GAPDH (representative results are shown in Fig. 5A, lanes 2–4) in a manner identical to anti-GAPDH mAb (Fig. 5A, lane 1). By contrast, negative controls, anti-U1 RNP and Sm standard serum YT (lane 5) and anti-Top I standard serum YM (lane 6) did not react with the 37 kDa GAPDH polypeptide.

We then tested for the presence of anti-GAPDH antibodies in various connective tissue diseases by an ELISA using the same antigen used in IB (Fig. 5B). We detected anti-GAPDH antibodies in 47% of SLE patients, which was a significantly \( P < 0.001 \) higher frequency than in patients with SjS (11%), SSc (10%), MCTD (10%) or RA (8%), or in normal control subjects (10%). This suggests anti-GAPDH antibody is a novel autoantibody strongly associated with SLE patients.

Analysis of the subcellular distribution of GAPDH using purified anti-GAPDH autoantibodies

GAPDH is known to be a glycolytic protein present in mitochondria, but our results showing an interaction with PCNA suggest a nuclear localization as well. We therefore analyzed the subcellular distribution of GAPDH using autoantibodies purified from SLE serum IS, which was reactive to GAPDH in immunoblots. Immunofluorescent staining using HEp-2 cells as a substrate showed nucleolar staining, speckled staining in the nucleoplasm and cytoplasmic staining (Fig. 6A). Anti-GAPDH antibodies purified from serum IS appeared to mainly stain the cytoplasm in a pattern resembled the staining obtained with anti-mitochondrial autoantibodies (Fig. 6B). Nevertheless, accumulation of GAPDH in nucleoplasm other than the nucleolus was also observed (Fig. 6B, white arrows and inset). Indeed, many cells showed speckled staining of the nucleus, though the staining was very

<table>
<thead>
<tr>
<th>Amino acid composition for unknown protein</th>
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<tr>
<td>PI: 9.13</td>
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<tr>
<td>Range: 8.88 - 9.38</td>
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<tr>
<td>MW: 39,700 Range: 31,760 - 47,640</td>
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<tr>
<td>Asx: 11.58</td>
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<tr>
<td>Glx: 7.52</td>
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<td>Pro: 2.29</td>
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<tr>
<td>Tyr: 6.93</td>
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<tr>
<td>Val: 9.04</td>
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<tr>
<td>Met: 0.10</td>
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<tr>
<td>Phe: 3.81</td>
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<td>Lys: 8.27</td>
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Fig. 3. Analysis of amino acid composition by ion-pair chromatography. Shown are the chromatography profile (A) and the amino acid composition of the protein deduced from the profile (B). Using the ExPASy protein database via World Wide Web servers, the protein was found to be identical to the 37 kDa GAPDH.

Fig. 4. Immunoblot showing that mouse anti-GAPDH mAb reacts with the 37 kDa polypeptide (white arrow) in TOB7–PCNA.

Fig. 5. (A) Immunoblot showing the reactivity of SLE sera to GAPDH. Eight sera reacted with the 37 kDa polypeptide in TOB7–PCNA in a manner identical to anti-GAPDH mAb: lane 1 anti-GAPDH mAb; lane 2, serum TT; lane 3, serum SH; lane 4, serum KY. By contrast, anti-U1 RNP and Sm standard serum YT (lane 5) and anti-Top I standard serum YM (lane 6) did not react (negative controls). (B) Using ELISAs in which serum anti-GAPDH antibodies levels were estimated based on absorbance at 405 nm, anti-GAPDH antibodies were detected in 47% of SLE patients, 11% of SjS patients, 10% of SSc and MCTD patients, 8% of RA patients and 10% of normal control subjects. The frequency among SLE patients was significantly higher than among patients with other connective tissue diseases or normal control subjects (\( P < 0.001 \)).
GAPDH almost completely disappeared from the serum (lanes 1 and 9–12), but when the disease subsequently flared, autoimmune response to the components of the complex spread again (lanes 13–17). Similar spread of the autoimmune response to various components of the PCNA complex was also observed in sera from anti-GAPDH-positive patient MS (Fig. 7C). Patient MS was diagnosed as having SLE because of positivity for ANA, oral ulcer, facial erythema, leukocytopenia and arthritis at the first visit of our hospital. At that time, serum MS was initially positive for only 37 kDa GAPDH (lane 1), but the autoimmunity spread to a 40 kDa protein (lane 2, weakly positive) and then to a 16 kDa protein, and proteins with higher mol. wts also started to appear. At the same time, the patient developed proteinuria although anti-dsDNA antibodies were negative. The spread of autoimmune response from the 37 kDa protein to proteins of the PCNA complex was also observed in anti-GAPDH positive sera from two other patients tested longitudinally (data not shown), and these data suggested that GAPDH plays a pivotal role in the induction of the autoimmune response to other components of the PCNA complex in some SLE patients positive for anti-GAPDH antibodies.

Fig. 6. Immunofluorescent staining showing the subcellular distribution of GAPDH. (A) Serum IS showed nucleolar staining, speckled staining of the nucleoplasm and cytoplasmic staining. (B) By contrast, purified anti-GAPDH antibodies from serum IS showed mainly cytoplasmic staining, though accumulation of GAPDH in the nucleoplasm (except the nucleolus) was observed in some cells (white arrow). Inset, higher magnification image of the indicated cell.

weak. Taken together, these data suggest that GAPDH is localizing not only in cytoplasm but also in nucleoplasm, and its accumulation likely occurs in association with cellular function and/or the cell cycle.

Longitudinal analysis of the reactivity of SLE serum with the PCNA complex

Because most PCNA-reactive SLE sera contain autoantibodies against several components of the PCNA complex, the reactivity of sera from patients showing GAPDH reactivity was longitudinally analyzed to study whether the spread of autoimmune responses within the complex could be detected. Initially, serum from patient SH who was diagnosed as having SLE because of positivity for antinuclear antibody (ANA) and anti-double stranded DNA (dsDNA) antibody, leukocytopenia and arthritis reacted mainly with 37 kDa GAPDH in TOB7-PCNA complexes involved in DNA replication and cell cycle regulation (21, 22). Although for over three decades GAPDH was considered a classical glycolytic enzyme involved in energy production (36), more recent evidence indicates that mammalian GAPDH has a number of other functions unrelated to its glycolytic activity (36). These include a role in membrane fusion, microtubule bundling, phosphotransferase activity, nuclear RNA export (36), and DNA replication and repair (36–38). In addition, other investigations suggest GAPDH is also involved in apoptosis, age-related neurodegenerative disease, prostate cancer and viral pathogenesis, and that these ‘new’ activities of GAPDH are related to its subcellular localization, oligomeric structure, relation to other proteins and kinetics in vivo (39). Among GAPDH’s many functions, DNA replication and repair are the most interesting to us because they parallel the functions of PCNA in nucleoplasm (5–9).

PCNA was first identified as an auxiliary protein of DNA Pol-δ, playing an essential role in the synthesis of the leading strand.

Discussion

Using 2D-PAGE and ion-pair chromatography, we have been able to show that GAPDH is an element of the PCNA complexes involved in DNA replication and cell cycle regulation (21, 22). Although for over three decades GAPDH was considered a classical glycolytic enzyme involved in energy production (36), more recent evidence indicates that mammalian GAPDH has a number of other functions unrelated to its glycolytic activity (36). These include a role in membrane fusion, microtubule bundling, phosphotransferase activity, nuclear RNA export (36), and DNA replication and repair (36–38). In addition, other investigations suggest GAPDH is also involved in apoptosis, age-related neurodegenerative disease, prostate cancer and viral pathogenesis, and that these ‘new’ activities of GAPDH are related to its subcellular localization, oligomeric structure, relation to other proteins and kinetics in vivo (39). Among GAPDH’s many functions, DNA replication and repair are the most interesting to us because they parallel the functions of PCNA in nucleoplasm (5–9).
during DNA replication (5,6). However, in vitro studies of SV40 DNA replication have led to a fuller understanding of the protein machinery, and it is now known that DNA replication is carried out by a very large multiprotein complex, the ‘DNA replication fork’, within which mammalian replication proteins such as RPA, replication factor C, Pol-δ, DNA polymerase α (Pol-α)/primase, RNase H, FEN-1, DNA ligase I and helicase all interact with PCNA during synthesis of both the leading and lagging strands (12,13). Moreover, when Baxi et al. analyzed GAPDH binding proteins, they found that GAPDH directly binds to Ap4A, which is known to be a regulator of DNA Pol-α and suggests the involvement of GAPDH in DNA replication (37,39). Although the molecular interaction of GAPDH and DNA Pol-α was reported, the association of GAPDH with DNA replication has not been well defined yet. Therefore, our results strongly supported the idea that GAPDH is involved in DNA replication interacting with PCNA.

We were able to detect the presence of GAPDH in nucleoplasm using purified autoantibodies against GAPDH. Although Singh and Green previously showed the presence of nuclear GAPDH associated with nuclear export of tRNA (40) and Ishitani et al. showed the accumulation of GAPDH in the nuclei of cells undergoing apoptosis (41), our observation represents the first report of nuclear localization of GAPDH detected by autoantibodies in SLE serum, suggesting the utility of this antibody as a probe for detecting GAPDH. Furthermore, nuclear distribution of GAPDH revealed by the speckled immunofluorescent staining in many HEp-2 cells supports the idea that GAPDH is involved in a variety of cellular functions ongoing in the nucleus. Our study also showed that GAPDH is a novel nuclear and cytoplasmic autoantigen in 47% of SLE patients, a significantly greater frequency than was seen in patients with other connective tissue diseases. Positivity for antinuclear antibodies is the most characteristic serological finding in SLE (42). Nonetheless, our data suggest that anti-cytoplasmic antibodies, including anti-GAPDH antibody, are also important serological makers for SLE, showing as high a frequency and specificity as anti-SSA/Ro, anti-ribosomal-P and anti-proteasome activator 28x antibodies (42,43).

In an earlier longitudinal study of the reactivity of SLE sera to PCNA complexes, we found that, with time, the immune response...
anti-GAPDH antibodies.

In this system. Closed circles indicate patients that were positive for

Borrelia burgdorferi

and TO17. Serum PCNA was detectable at concentrations

6 ng/ml.

response spread among the elements of the PCNA complexes

(23). Those results suggested that 'intermolecular–intrastructural
help' plays a key role in the induction of autoimmune responses
to PCNA complexes (23,44). It is known that for such
intermolecular–intrastructural help to occur, tolerance to one
of the elements of a macromolecular complex must be lost, but
it was not known which element(s) initiated the spread of the
autoimmune response within PCNA complexes. Our results
suggest GAPDH is one of the possible proteins that can play
a pivotal role in the induction of the autoimmune response. This
idea is supported by the findings that the autoimmune
response spread from the 37 kDa GAPDH polypeptide to other
components of the PCNA complex in a longitudinal study
(Fig. 7), and that serum PCNA levels, which may be crucial for
'antigen presentation', are elevated in a significantly larger
fraction of SLE patients positive for anti-GAPDH antibodies
than in those negative for the antibody (Fig. 8). Consistent with
these findings, our earlier studies showed significant increases
in both PCNA-positive PBMCs, mainly T cells (45,46), and
serum PCNA levels (21,33–35), suggesting that, in SLE
patients, PCNA complexes are released into the peripheral
blood along with other nuclear antigens when activated cells
are killed by apoptosis (47,48).

It is also possible that the autoimmune response to GAPDH
is initiated by T and B cells reacting to 'foreign-GAPDH'
(49–56). GAPDH has been shown to be present on the cell
surfaces of parasites such as Schistosoma mansoni (49–51),
bacteria such as group A Streptococci and Staphylococcus
aureus (52,53), fungi such as Candida albicans (55), and
Borrelia burgdorferi, the Lyme disease agent (56); and to
induce anti-GAPDH antibodies (49–51,54–56). In addition,
GAPDH is a selective target for antibodies in sera from
humans resistant to reinfection of S. mansoni (49–51), and an
antigenic determinant on GAPDH has been used in a subunit
vaccine (51). It is also known that Brucella GAPDH can induce
the production of anti-GAPDH antibodies in cattle, sheep and
mice (54), and DNA immunization with GAPDH gene plus
murine interleukin-12-expressing plasmid DNA can induce
partial protection against Brucella infection in mice (54). All of
these results are indicative of the strong antigenicity of GAPDH
during infections, and that it can be used as a target antigen
for vaccine therapy. This means that if GAPDH is released into
the serum of patients having an immune response to 'foreign
GAPDH' induced by an infection, self-GAPDH might induce
autoantibody production, as the structure of GAPDH is highly
conserved among species—e.g. if a conserved sequence
such as GFGRIG (residues 8–13), known to be an ATP binding
site, is antigenic (39,56). Of course, many subjects tested
for anti-GAPDH antibodies may have immune responses
to foreign GAPDH; indeed low levels of anti-GAPDH antibodies
were detected in ~10% of normal control subjects, but the
increased level of serum PCNA that interacts with GAPDH is
only seen in SLE patients. Consequently, specific production
of autoantibodies against GAPDH and the spread of the
autoimmune response to other elements of the PCNA complex
can only occur in SLE patients. For that reason, along with the
finding that bacterial DNA having CpG residues can induce
production of anti-DNA antibodies, a possible role for bacterial
infection in the induction of SLE has been postulated (57,58).
Our results suggest yet another mechanism by which bacterial
or other infections may induce autoimmune responses in SLE
patients, although we need further studies to confirm this issue.

In sum, our findings demonstrate that the PCNA complex
contains GAPDH targeted as an autoantigen in SLE patients
and highlight the importance of analysis of PCNA complexes
for understanding the mechanisms by which autoimmune
responses are induced in connective tissue disease.

Acknowledgements

Supported in part by a Grant-Aid for Scientific Research of Ministry of
Education and Science (No. 13670476).

Abbreviations

ANA antinuclear antibodies
CDK cyclin-dependent kinase
DID double immunodiffusion
dsDNA double stranded DNA
IB immunoblot
MCMT human DNA-(cytosine-5) methyltransferase
MCTD mixed connective tissue disease
NDH II DNA helicase II
PCNA proliferating cell nuclear antigen
PCNA complex PCNA multiprotein complex
PM/DM polymyositis/dermatomyositis
RA rheumatoid arthritis
RPA replication protein A
RTE rabbit thymus extract
SjS Sjögren’s syndrome
SLE systemic lupus erythematosus
Top I topoisomerase I
TTF-1 transcription terminator factor 1
1304  Autoimmune response to GPDH in lupus patients


