Partial Proteolysis of Simian Virus 40 T Antigen Reveals Intramolecular Contacts between Domains and Conformation Changes upon Hexamer Assembly

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Simian virus 40 large tumor antigen (Tag) is a multifunctional viral protein that binds specifically to SV40 origin DNA, serves as the replicative DNA helicase, and orchestrates the assembly and operation of the viral replisome. Tag associated with Mg-ATP forms hexamers, and in the presence of SV40 origin DNA, double hexamers. Limited tryptic digestion of monomeric Tag revealed three major stable structural domains. The N-terminal domain spans amino acids 1–130, the central domain comprises amino acids 131–476, and the C-terminal domain extends from amino acid 513 to amino acid 698. Co-immunoprecipitation of digestion products of monomeric Tag suggests that the N-terminal domain associates stably with sequences located in the central region of the same Tag molecule. Hexamer formation protected the tryptic cleavage sites in the exposed region between the central and C-terminal domains. Upon hexamerization, this exposed region also became less accessible to a monoclonal antibody whose epitope maps in that region. The tryptic digestion products of the soluble hexamer and the DNA-bound double hexamer were indistinguishable. A low-resolution model of the intramolecular and intermolecular interactions among Tag domains in the double hexamer is proposed.

* This work was supported by grants from the Deutsche Forschungsgemeinschaft (to H.-P. N. and F. G.), National Institutes of Health Grant GM52948 (to E. F.), and grants from the Howard Hughes Medical Institute Professors Program (to E. F.), and Vanderbilt University Grant GM52948 (to E. F.), and grants from the Howard Hughes Medical Institute Professors Program (to E. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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§ The abbreviations used are: Tag, large tumor antigen; Pab, papova-virus monoclonal antibody; AMP-PNP, adenosine 5’-(β, γ-imido)triphosphate; RPA, replication protein A.

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Published, JBC Papers in Press, July 9, 2004, DOI 10.1074/jbc.M406159200

This paper is available on line at http://www.jbc.org

Printed in U.S.A.

Received for publication, June 3, 2004

07745 Jena, Germany.

Printed in U.S.A.

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Intramolecular Contacts between T Antigen Domains

EXPERIMENTAL PROCEDURES

**Limited Proteolysis of Tag Monomers and Analysis of Digestion Products**—Tag expressed in Sf9 cells infected with a recombinant baculovirus was purified as described (23). Sequencing grade trypsin (Roche Applied Science) (dissolved and diluted in 1 mM HCl) and 5 μg of Tag (0.94 μg/pmol) in the indicated protease/Tag mass ratios were incubated in a 10-μl reaction mixture containing 20 mM Hepes-KOH, pH 8, 10% glycerol, and 3 mM dithiothreitol. After 30 min at room temperature, trypsin was inhibited by the addition of 1 μl of a 0.4 mg/ml solution of α-phenylmethylsulfonyl fluoride (Roche Applied Science) to yield a final concentration of ~40 μg/ml. Incubation was continued for 2 min on ice before the addition of 4 μl of 4× Laemmli sample buffer (24). The samples were electrophoresed on 12.5% denaturing polyacrylamide gels. The gels were either stained with Coomassie or immunoblotted (25). Blots were probed with Tag-specific monoclonal antibodies using chemiluminescence (ECL; Amersham Biosciences). Epitope mapping and characterization of the Tag-specific monoclonal antibodies Pab108, Pab101, Pab419, Pab416, Pab414, Pab220, Pab221, Pab204, and KT3 have been described (Ref. 26 and references therein). Using a peptide library kindly provided by Jens Schneider-Mergener (Jerini AG, Berlin, Germany), the epitope for Pab101 was mapped to amino acids 696–708 (data not shown).

**Hexamer Formation**—Five micrograms of Tag were incubated for 30 min at 37 °C in a 10-μl reaction mix containing 30 mM Hepes-KOH, pH 7.8, 1 mM dithiothreitol, 4 mM AMP-PNP (Sigma-Aldrich), and 7 mM MgCl2. In some experiments, 1 pmol/μl of 7 mM MgCl2. In some experiments, 1 pmol/μl of a 100-kDa marker proteins (m) (lanes 1 and 7) were electrophoresed in parallel. The positions of Coomassie-stained full-length Tag (7) and proteolytic fragments, numbered in order according to their electrophoretic mobility, are indicated on the right.

bands of interest were excised, and the first 8–10 N-terminal amino acids were determined using a Procise 494 HT Protein Sequencer (Applied Biosystems, Weiterstadt, Germany).

**Immunoprecipitation of Monomeric and Hexameric Tags**—Ten micrograms of purified monoclonal antibody were bound to 20 μl of 50% (v/v) protein G-agarose beads in 100 μl of binding buffer (50 mM Hepes-KOH, pH 7.9, 100 mM KC1, 7 mM MgCl2, 0.25% inositol, 0.25 mM EDTA, and 0.05% Nonidet P-40) for 1 h at 4 °C. The beads were then washed four times with 1 ml of wash buffer (30 mM Hepes-KOH, pH 7.9, 100 mM KC1, and 7 mM MgCl2). One picomole of Tag (0.94 μg/pmol) preincubated for 30 min at room temperature in 100 μl of binding buffer (monomeric Tag) or binding buffer supplemented with 7 mM MgCl2 and 4 mM AMP-PNP (hexameric Tag) was added to the beads. After 1 h at 4 °C, the beads were washed four times with 1 ml of wash buffer and then boiled in 20 μl of 2× Laemmli sample buffer. Eluted proteins were electrophoresed on a 7.5% denaturing polyacrylamide gel and detected by Western blotting (25) using a mixture of Pab419 and 101 with the ECL detection system (Amersham Biosciences). The amount of precipitated Tag was evaluated by densitometry of the exposed films.

**Immunoprecipitation after Trypsin Treatment**—Ten microliters of the digestion products, 20 μl of 50% (v/v) protein G-agarose (Roche Applied Science), and 10 μg of either monoclonal antibody Pab419, specific for the N terminus, or Pab204, directed against the central region, were added to 500 μl of immunoprecipitation buffer (20 mM Hepes-KOH, pH 7.9, 150 mM NaCl, and 0.5% Nonidet P-40) supplemented with 0.05% SDS/0.1% sodium deoxycholate. After incubation at 4 °C for 1 h with shaking, the beads were washed four times with supplemented immunoprecipitation buffer and once with immunoprecipitation buffer. The beads were then boiled in 2× Laemmli sample buffer. After gel electrophoresis and transfer to nitrocellulose filters, protein fragments were detected by Western blotting with Pab419 or Pab204 and the ECL system. Alternatively, digestion products were first denatured by adding 10% SDS and 100 mM dithiothreitol to a final concentration of 1% and 10 mM, respectively, heated at 90 °C for 2 min, cooled on ice, and then immunoprecipitated with Pab419 or Pab204 as described above.

**Electrophoretic Mobility Shift**—Two fragments of the SV40 origin-containing 5′-end-labeled EcoRI-HindIII DNA fragment of pUCmori (26) (specific activity 10,000 cpm/fmol) were incubated in a 10-μl reac-
Untreated Tag (lanes 3) of loading buffer (10 mM Hepes-KOH, pH 7.8, 2% Ficoll 400, 0.2% of 0.2% and incubating for 5 min, followed by the addition of 0.2 volumes cross-linked to DNA by adding glutaraldehyde to a final concentration assay 10 min after the Tag was added (post-incubation). Protein was (pre-incubation), or 1 °C albumin) with 30 fmol of Tag for 30 min at 37 digestion with trypsin (t), lanes 2 at a protease/protein mass ratio of 1:500 (lanes 10, 11, 12, 14, 16, 18, and 20) and separated by 13.5% SDS-PAGE. Untreated Tag was readily degraded to fragments of 88, 77, 70, and 55 kDa in apparent molecular mass (Fig. 1, Tag was either pre-incubated with 1 °C albumin before its addition to the DNA binding reaction was either pre-incubated with 1 °C albumin (pre-incubation), or 1 µg of antibody was added to the DNA binding assay 10 min after the Tag was added (post-incubation). Protein was cross-linked to DNA by adding glutaraldehyde to a final concentration of 0.2% and incubating for 5 min, followed by the addition of 0.2 volumes of loading buffer (10 mM Hepes-KOH, pH 7.8, 2% Ficoll 400, 0.2% bromphenol blue, and 0.2% xylene cyanol). Protein-DNA complexes were resolved by electrophoresis in a 3.5% native polyacrylamide gel in TBE (89 mM Tris, 89 mM boric acid, and 1 mM EDTA) at 200 V. The gel was dried and autoradiographed, and DNA binding was quantified by densitometry of the autoradiogram.

RESULTS

Limited Proteolysis of Tag and Mapping of Tryptic Fragments—Monomeric Tag was treated with increasing amounts of trypsin, and the digestion products were analyzed by denaturing gel electrophoresis (Fig. 1). Full-length Tag with a calculated molecular mass of 82.5 kDa migrates as a band with an apparent molecular mass of 94 kDa (Fig. 1, lane 1, fragment T). Tag was readily degraded to fragments of 88, 77, 70, and 55 kDa in apparent molecular mass (Fig. 1, lane 3, fragments 1-4 and 6). Increasing the protease concentration yielded additional fragments of 56, 53, 45, 40, 28, 25, 17, 15, and 13 kDa (Fig. 1, lane 4, fragments 5, 8–14). When the protease concentration was raised further, the yield of fragments 1–6 decreased significantly, whereas that of fragments 8–14 remained constant (Fig. 1, lane 5). Even at the highest protease concentration tested, fragments 8–14 were resistant to proteolysis (Fig. 1, lane 6).

The approximate location of each fragment in the primary Tag sequence was mapped by immunoblotting the digestion products using monoclonal antibodies with known epitopes (Fig. 2). To this end, Tag was digested at a protease/protein ratio of 1:500, yielding all of the tryptic fragments 1–14 detected in Fig. 1 (Fig. 2, lane 2, t). Each antibody reacted with the full-length, undigested Tag as expected (Fig. 2, odd-numbered lanes, u). Fragments 1, 5, 6, 12, 13, and 14 contained the N-terminal part of Tag, because they reacted with the N-terminal specific antibodies Pab108, Pab419, and Pab416 (Fig. 2, lanes 4, 6, and 8). Fragments 3–6, 8, and 9 were detected by Pab204, demonstrating that they contained the central part of Tag (Fig. 2, lane 14). Fragments 5 and 6 were reactive to both N-terminal and central region antibodies. However, because their length was too short to allow recognition by both antibodies, the data imply that the positions marked 5 and 6 (Fig. 2, far left) must contain a mixture of fragments that will be referred to as 5a and 5b and 6a and 6b (Table I). Fragment 2 reacted with all antibodies except those against the extreme C terminus, indicating that this fragment lacks the C terminus of Tag (Fig. 2, lanes 18 and 20) and possibly some N-terminal residues. Fragment 7 was detected by antibodies Pab416, Pab220, and Pab221, but not Pab204 (Fig. 2, lanes 8, 10, 12, and 14), suggesting that it was derived from the central part of Tag on the N-terminal side of the Pab204 epitope. Judging by their
TABLE I
Mapping data of tryptic fragments of Tag

| Fragment designation | Size in kDa | Antibody reactivity | N terminal sequence | N terminal boundary | C terminal boundary | N terminal\(^a\) potential cleavage | C-terminal\(^a\) potential cleavage |
|----------------------|-------------|---------------------|---------------------|--------------------|---------------------|-------------------------------|-------------------------------|
| T                    | 94          | 108, 419, 416, 220, 221, 204, 414, 101, KT3 | N. D.              | 1 (C)              | 708 (A)             | 1, Lys-2, Arg-5             | 708                           |
| 1                    | 88          | 108, 419, 416, 220, 221, 204, 414 | N. D.              | 1 (A)              | 698 (C)             | 1, Lys-2, Arg-5             | Lys-691, Lys-697, Lys-698 |
| 2                    | 77          | 204, 414            | N. D.              | 131 (C)            | 698 (A)             | Arg-130                      | Lys-691, Lys-697, Lys-698 |
| 3                    | 70          | 204                 | \(^{131}\)KVEDPKD\(^{137}\) | 131 (S)            | 652 (C)             | Arg-130                      | Lys-652                      |
| 4                    | 63          | 204                 | \(^{131}\)KVEDPKD\(^{137}\) | 131 (S)            | 605 (C)             | Arg-130                      | Arg-602, Lys-605, Lys-614, Lys-616 |
| 5a                   | 56          | 108, 419, 416       | N. D.              | 1 (A)              | 420 (C)             | 1, Lys-2, Arg-5             | Lys-410, Lys-418, Lys-419, Lys-420 |
| 5b                   | 56          | 204                 | \(^{131}\)KVEDPKD\(^{137}\) | 130 (S)            | 554 (C)             | Arg-130                      | Arg-554, Lys-566, Arg-567 |
| 6a                   | 55          | 108, 419, 416       | N. D.              | 1 (A)              | 418 (C)             | 1, Lys-2, Arg-5             | Lys-410, Lys-418, Lys-419, Lys-420 |
| 6b                   | 55          | 204                 | \(^{131}\)KVEDPKD\(^{137}\) | 131 (S)            | 543 (C)             | Arg-130                      | Arg-535, Lys-543, Arg-546, Arg-550 |
| 7                    | 53          | 416, 220, 221       | N. D.              | 68 (C)             | 456 (A)             | Lys-51, Lys-53, Lys-54, Lys-60, Lys-61, Lys-67 | Arg-456 |
| 8                    | 45          | 204                 | \(^{131}\)KVEDPKD\(^{137}\) | 131 (S)            | 483 (A)             | Arg-130                      | Lys-476, Lys-483 |
| 9                    | 40          | 204                 | \(^{131}\)KVEDPKD\(^{137}\) | 131 (S)            | 476 (A)             | Arg-130                      | Lys-476, Arg-483 |
| 10                   | 28          | None                | \(^{486}\)DLPSGQG\(^{500}\) | 484 (S)            | 698 (C)             | Arg-483                      | Lys-691, Lys-697, Arg-698 |
| 11                   | 25          | None                | \(^{513}\)HLNKRTG\(^{519}\) | 513 (S)            | 698 (C)             | Lys-512                      | Lys-691, Lys-697, Arg-698 |
| 12                   | 17          | 108, 419, 416       | N. D.              | 1 (C)              | 130 (A)             | 1, Lys-2, Arg-5             | Lys-127, Lys-128, Lys-129, Arg-130, Lys-136 |
| 13                   | 15          | 108, 419, 416       | N. D.              | 22 (C)             | 130 (A)             | Lys-21                       | Lys-127, Lys-128, Lys-129, Arg-130, Lys-136 |
| 14                   | 13          | 108, 419, 416       | N. D.              | 33 (C)             | 130 (A)             | Arg-31, Lys-32, Lys-36, Lys-37, Lys-39, Lys-45 | Lys-127, Lys-128, Lys-129, Arg-130, Lys-136 |

\(^a\) One or more alternatives for the calculated cleavage sites.

mapping data of tryptic fragments of Tag

The meaning of the letters in parentheses in the N terminus boundary and C terminus columns are as follows: A, deduced from antibody reactivity and peptide size; C, calculated from apparent molecular mass of the peptide that the position of one terminus; S, obtained from peptide sequencing. N. D. under the N terminal sequence column is “not determined.”

- Sizes and reactivity with Pab204, fragments 3, 4, 5a, 5b, 6a, 6b, 8, and 9 should contain the epitopes of Pab 220 and Pab221. However, these antibodies, like Pab414, are conformation-specific and do not react with denatured fragments (26). Thus, fragments 2 and 7 retained these conformational epitopes to some extent. Fragments 10 and 11 did not react with any of the antibodies used.

To map the N-terminal ends of the tryptic fragments more precisely and identify the derivation of fragments 10 and 11, Coomassie-stained bands were subjected to N-terminal sequencing. The N termini of fragments 3, 4, 5b, 6b, 8, and 9 were all derived from a single prominent cleavage site after arginine 130 (Table I). Fragments 10 and 11 were derived by cleavage after arginine 483 and lysine 512, respectively. T antigen and fragments 1, 5a, 6a, and 12 yielded no N-terminal sequence information, probably due to an N-terminal modification (1–3). Fragments 7, 13, and 14 yielded sequence data that could not be interpreted.

Based on peptide size, antibody reactivity, and N-terminal sequence, we were able to fit each fragment into a tryptic map (Fig. 3). For each fragment for which N-terminal sequence information was available (fragments 3, 4, 5b, 6b, 8, 9, 10, and 11), the most likely C-terminal cleavage site was calculated according to the apparent size of the fragment (Table I). Because fragment 1 did not react with KT3 or Pab101 and fragments 5a and 6a did not react with Pab204, but all of them reacted with N-terminal antibodies, it was reasonable to propose that they all contained the N terminus of Tag, allowing us to calculate their most likely C-terminal cleavage sites based on the fragment sizes. The small fragments 12, 13, and 14 were clearly N-terminal based on their antibody reactivity with Pab108, Pab419, and Pab416, but based on their apparent sizes, the absence of potential cleavage sites for trypsin between amino acids 68 to 126, and the strong cleavage site after Arg-130, we reasoned that fragments 12, 13, and 14 ended with amino acid 130. This led us to map fragment 12 to residues 1–130 and fragments 13 and 14 to the same region, but with N-terminal truncations. Fragment 2 reacted exclusively with antibodies whose epitopes map in the central part of the protein. Based on the size of peptide 2 and absence of reactivity with Pab101 and KT3, the extreme C terminus was assumed to be missing as for fragment 1, and, accordingly, its N terminus could be determined by calculation. Fragment 7 did not react with Pab419 or Pab204, placing it between these two epitopes. We wish to stress that because of uncertainties in peptide size calculations and the imprecise mapping of many of the antibody epitopes, the calculated termini of the fragments shown in Fig. 3 must be considered as approximations. Table I lists alternative potential alternative cleavage sites that are consistent with apparent size, immunoreactivity, and available N-terminal sequence.

Co-immunoprecipitation of Complexes between Protease-resistant Fragments of Tag Monomers—To search for intramolecular interactions among Tag domains, we reasoned that if
such contact surfaces between domains were sufficiently stable after tryptic digestion of native monomeric Tag, we might be able to detect them by co-immunoprecipitation. Because it was of particular interest to identify contacts of the N-terminal domain with the central or C-terminal region in order to extend our understanding of full-length Tag structure, we chose monoclonal antibodies with epitopes in the N-terminal (Pab419) and central regions (Pab204) to precipitate Tag digests (see the epitope map in Fig. 2). An antibody, 70C, which does not react with Tag (27), was used as a negative control. Immunoprecipitation was carried out using tryptic digests of Tag, either before denaturation to detect co-precipitated fragments or after denaturation as a control for the specificity of the pull-down (Fig. 4).

When the input tryptic digests were immunoblotted with Pab204, fragments 2, 3, 4, 5b, 6b, 8, and 9 were visible (Fig. 4, lanes 1 and 5), consistent with the peptide mapping in Figs. 2 and 3. When the native digest was precipitated with Pab419 and blotted with Pab204, fragments 2, 4, 8, and 9 were still detected in the precipitate (Fig. 4, lane 3). In contrast, when the digest was denatured before immunoprecipitation with Pab419, fragments 4, 8, and 9 were no longer detectable by Pab204 (Fig. 4, lane 4). We conclude from this result that fragments 4, 8, and 9 were associated with an N-terminal domain of Tag in the native digest. Conversely, when either the native or the denatured digest was precipitated with Pab204 and stained with the same antibody, the same set of fragments 2, 3, 4, 5b, 6b, 8, and 9 were visible both with and without denaturation before immunoprecipitation (lanes 7 and 8).

When the input tryptic digest was immunoblotted with Pab419, fragments 1, 5a, 6a, 12, 13, and 14 were detected (Fig. 4, lanes 9 and 13). When the digest was immunoprecipitated with Pab419, either before or after denaturation, and immunoblotted with Pab419, only fragments 1 and 12–14, which contain the 419 epitope, were precipitated (Fig. 4, lanes 11 and 12). For unknown reasons, fragments 5a and 6a, which are recognized by Pab419 in the input digest (Fig. 4, lane 9), were either masked by the antibody heavy chain or not precipitated (Fig. 4, lanes 11 and 12). Immunoprecipitation of the native digest with Pab204, followed by staining with Pab419, identified fragments 1, 5a, 6a, and 12 (Fig. 4, lane 15). Because peptides 5a, 6a, and 12 do not contain the Pab204 epitope, we conclude that they co-precipitated with peptide 1 (Fig. 4, lane 15). These N-terminal fragments were not observed when the digest was denatured prior to immunoprecipitation with Pab204, demonstrating that the Pab204 antibodies did not recognize the denatured N-terminal fragments (Fig. 4, lane 16). No Tag fragments were precipitated by the negative control antibody 70C (Fig. 4, lanes 2, 6, 10, and 14), demonstrating the specificity of the immunoprecipitation.

**Limited Proteolysis of Tag Hexamers and Double Hexamers**—To assess whether hexamer formation affected the proteolytic digestion pattern, Tag was first incubated in the presence of magnesium chloride and AMP-PNP to induce oligomerization. The input Tag was >95% monomeric and accompanied by a small amount of dimers (Fig. 5A, lane 2). In the presence of magnesium alone, dimeric Tag increased, and small amounts of higher oligomeric forms were observed (lane 3). AMP-PNP by itself induced oligomerization to a lesser extent (Fig. 5, lane 4).
but in the presence of both magnesium and AMP-PNP the Tag monomers were quantitatively converted into hexamers and a smaller amount of dodecamers (Fig. 5, lane 5). Exposure of Tag to SV40 origin DNA alone induced some dimer formation (Fig. 5, lane 6), but hexamers and dodecamers were predominant when magnesium and AMP-PNP were also present (Fig. 5, lane 7).

Using each of the conditions established in Fig. 5A, Tag was then digested with trypsin at a protease/protein ratio of 1:250 (see Fig. 1, lane 5). In the absence of magnesium, AMP-PNP, and origin DNA, the familiar proteolytic pattern was obtained (Fig. 5B, lane 2). The addition of any one of these components led to a clear decrease in fragments 8–11 (Fig. 5B, lanes 3, 4, and 6). This reduction in fragments 8–11 was correlated with
the fraction of Tag in higher oligomeric forms, with the greatest effect in the presence of magnesium and the least effect in the presence of origin DNA (compare Fig. 5, A with B). Fragments 8–11 were completely absent when both magnesium and AMP-PNP were present, with or without origin DNA, and the amount of fragment 4 was clearly increased (Fig. 5B, lanes 5 and 7). More subtle effects on the abundance of fragments 2, 3, 5, and 6 were also observed (Fig. 5B, lanes 5–7). The results suggest that the accessibility of the central region of Tag to tryptic digestion decreases quite strongly upon hexamer assembly, either in solution or on origin DNA.

Binding of Region-specific Antibodies to Monomeric and Hexameric Tag—The Tag epitope recognized by antibody Pab204 falls in the central region of Tag in the general vicinity of the tryptic cleavage sites that are accessible in Tag monomers and protected in hexamers. This coincidence raised the question of whether the epitopes for Pab204 or the other Tag-specific monoclonal antibodies used in Fig. 2 may be differentially accessible in the monomeric and hexameric forms of the protein. To address this question, we pre-incubated each antibody with Tag, either with monomers before double hexamer assembly on SV40 origin DNA or after assembly, and analyzed the complexes in electrophoretic mobility shift assays (Fig. 6). In the absence of Tag, no mobility shift of a radiolabeled double-stranded origin DNA fragment was observed (Fig. 6A, A and B, lanes 1). The addition of Tag shifted most of the DNA into the double-hexameric complex (Fig. 6A, A and B, lanes 2). The presence of antibody buffer did not alter this result (Fig. 6A, A and B, lanes 3). When the antibodies were incubated with Tag monomers prior to double hexamer assembly, most of the antibodies had no effect on the assembly of the double hexameric complex and remained bound to the complex, detected as a supershift in the mobility of the double hexamer (Fig. 6A, lanes 4–7 and 9–11). Similarly, most of the antibodies were able to bind to the double hexamer complex after assembly on origin DNA, resulting in a supershift (Fig. 6B, lanes 4–7 and 9–11). We note, however, that the supershifted complex generated with Pab220 and Pab221 migrated slightly faster than those generated by the other antibodies (Fig. 6, A and B, lanes 6 and 7). It is possible that the double hexamer binds fewer Pab220/221 antibody molecules than what was observed with the other antibodies.

In a major exception to these results, pre-incubation of Tag monomers with antibody Pab204 inhibited double hexamer assembly completely (Fig. 6A, lane 8). When Pab204 was added to Tag double hexamers after assembly on DNA, it destabilized the double hexamer, resulting in a range of supershifted and single hexameric complexes bound to origin DNA (Fig. 6B, lane 8). The destabilizing effect of Pab204 on DNA-bound Tag double hexamers could be caused by a reduction in either origin DNA binding or hexamer assembly. To distinguish between these possibilities, we compared the amounts of monomeric and hexameric Tag immunoprecipitated by each antibody. All of the antibodies except Pab204 precipitated both forms of Tag about equally well (Fig. 6C, lanes 1–16). Pab204, on the other hand, precipitated the hexameric form about half as well as it did the monomeric Tag (compare lanes 9 and 10), indicating limited accessibility of the Pab204 epitope in the hexamer.

**DISCUSSION**

**Structural Domains of SV40 Tag**—Limited tryptic digestion of baculovirus-expressed Tag reveals three major protease-resistant fragments with apparent molecular masses of 17, 40–45, and 25–28 kDa (Fig. 1). The tryptic fragments of Tag were mapped in the primary sequence by immunoblotting with monoclonal antibodies that have known epitopes, N-terminal sequencing, and deduction based on apparent peptide size and the location of potential tryptic cleavage sites (Figs. 2 and 3 and Table I). We did not observe any fragments containing the true C terminus of Tag (Fig. 2), suggesting that the cleavage site at Arg-698 must be very exposed, consistent with its known susceptibility to proteolysis during Tag purification (28, 29).

The three major protease-resistant fragments mapped in Tag define the three structural domains, I, II, and III, that are diagrammed in Fig. 7A. We subdivided domain II into two parts (a and b) based on a Pronase E-resistant fragment generated with origin DNA-bound Tag (30) and the atomic structure of residues 131–259 (21). Monomeric Tag contains an
extremely protease-sensitive region in the C-terminal part of domain IIb (darker shading in Fig. 7A). Although many of these tryptic cleavage sites have been observed in previous proteolysis studies performed with Tag expressed in mammalian cells (reviewed in Refs. 1–3), the stable fragment 513-698 (domain III in Fig. 7A), detected here and independently by Chen and colleagues (31), is novel. The sequenced N termini reported here are completely consistent with those determined by Chen and colleagues (31). Our use of monoclonal antibodies whose epitopes are small and well mapped affords somewhat greater resolution of the multiple cleavage sites in the C terminus of domain IIb and the extreme C terminus of Tag (Fig. 2). Minor differences in deduced cleavage sites based on the apparent molecular mass of peptides are probably due to technical differences between the two studies (31).

The protease-resistant domains in Fig. 7A correspond closely to the Tag domains whose atomic structures have now been solved, i.e. domain I (22), domain IIa (21), and domain IIb-III (20). They also correspond well to functional domains of Tag that have been defined through biochemical analysis of deletion mutants in many laboratories, as summarized in Fig. 7B (20–22) (reviewed in Refs. 1–3, 32, and 33). Domain I of Tag contains the DnaJ domain, which binds to Hsc70 and plays a role in facilitating the ubiquitylation and destruction of the retinoblastoma family proteins that bind in the C-terminal region of Tag domain I (Fig. 7B, dotted line) (32, 33). The nuclear localization signal of Tag and a cluster of phosphorylation sites that modulate interactions between Tag hexamers to promote bidirectional DNA unwinding during viral replication are also located in the C-terminal region of domain I (1–3, 5, 6). Domain IIa is multifunctional, being sufficient for origin DNA binding activity, binding to host RPA, topoisomerase I, transcription factors, and perhaps other proteins (1, 2, 3). Domain IIb-III contains the helicase activity of Tag (20, 31) as well as binding sites for the tumor suppressor protein p53, DNA polymerase α-primase, and other host proteins (1–3, 33). In addition, a role for domain III in contacting origin DNA in the double hexamer was recently reported (34). The protease-sensitive C terminus of Tag harbors a poorly understood host
range function needed for virus production in some host cells (1–3).

**Intramolecular Contacts between Domains I and II**—The available atomic structures and electron microscopic data on native Tag and its oligomeric forms do not provide information on the location of domain I (13–22). By immunoprecipitating a native tryptic digest of Tag monomers using a monoclonal antibody against an epitope in domain IIb (Pab204), short fragments containing only domain I were detected in the precipitate, implying that the short domain I fragments remained associated with domain II after digestion. In support of this interpretation, Pab204 did not immunoprecipitate domain I fragments from a denatured digest (Fig. 4). When the converse experiment was performed using an antibody reactive with domain I for immunoprecipitation (Pab419), three fragments that contained domain II, but not domain I, immunoprecipitated (Fig. 4). Taken together, the data strongly suggest that domain I associates with domain II in native Tag, even when the polypeptide backbone connecting the two domains is cleaved. Because antibodies specific for domain III were not used, we cannot assess whether domain I may also interact with domain III. This interpretation is consistent with the independent observation that tryptic fragments of Tag from domains I, II, and III form complexes that remain stable through gel filtration (31). This intramolecular interaction between the N terminus and the central region of Tag also provides a plausible explanation for the previously observed requirement for domain IIB of Tag to permit casein kinase I to phosphorylate its targets in domain I (Ser-120 and Ser-123) (35). The ability of the J domain to associate with domain II raises new and interesting questions about whether the J domain function of Tag may remodel protein-protein interactions that involve domain IIA and IIB, akin to those involving retinoblastoma-related proteins (32, 33). Among the potential candidates for remodeling by the J domain are p53, DNA polymerase, and hectorin, which are phosphorylated by casein kinase I in a peptide encompassing domains I and IIA but were phosphorylated in full-length Tag (35). Interactions between Tag monomers in a hexamer, with or without origin DNA, were demonstrated to mask tryptic cleavage sites in domain IIB and to be perturbed by Pab204 binding in IIB (Figs. 5B and 6). Domain IIB interactions between Tag monomers mediate hexamer formation in the atomic structure of the helicase domain and are critical for helicase activity, whereas domain IIIB is not necessary for hexamer assembly or helicase activity (8, 31). We also propose interactions between Tag monomers through domain I based on its importance in hexamer assembly (36). Lastly, hexamer-hexamer interactions are mediated by domain IIA and modulated directly or indirectly by domain I (5, 10, 12, 34, 36). Future work to complete the atomic structure of full-length Tag and further characterize the double hexamer assembly on origin DNA, coupled with biochemical studies, will test this model and provide insight into the functional roles of the proposed interactions.

**Acknowledgments**—We thank J. Schneider-Mergener for the gift of a plasmid library, X.-S. Chen for communication of data prior to publication and stimulating discussion, and E. M. Warren for expert technical assistance.

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Intramolecular Contacts between T Antigen Domains

38951
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J. Biol. Chem. 2004, 279:38943-38951.
doi: 10.1074/jbc.M406159200 originally published online July 9, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406159200

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