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Polyoma middle-sized T antigen can be phosphorylated on tyrosine at multiple sites *in vitro*

Tony Hunter*, Mary Anne Hutchinson and Walter Eckhart

Molecular Biology and Virology Laboratory, The Salk Institute, Post Office Box 85800, San Diego, CA 92138, USA

*To whom reprint requests should be sent

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The polyoma middle-sized T antigen (MT antigen) is associated with a protein kinase activity which phosphorylates tyrosine residues in polyoma T antigens *in vitro*. We have studied the sites of tyrosine phosphorylation of MT antigens phosphorylated in immunoprecipitates or in soluble form after partial purification by immunoaffinity chromatography. By analyzing the amino acid sequences of tryptic peptides of MT antigen, and by analyzing deletion mutant MT antigens, we have identified two major sites of phosphorylation in MT antigen, tyrosines 250 and 315. Additional sites were phosphorylated under some conditions. A synthetic peptide (Glu.Glu.Glu.Glu.Tyr.Met.Pro.Met.Glu), corresponding to the sequence around tyrosine 315, was phosphorylated when added to immunoprecipitates containing MT antigen.

Key words: phosphorylation/polyoma virus/protein kinase/T antigens

Introduction

The polyoma middle-sized tumor antigen (MT antigen) is associated with a protein kinase activity in immunoprecipitates (Eckhart *et al.*, 1979; Smith *et al.*, 1979; Schaffhausen and Benjamin, 1979). The acceptor amino acid in MT antigen proves to be exclusively tyrosine (Eckhart *et al.*, 1979). Partially purified soluble preparations of the MT antigen also possess this phosphotransferase activity (Walter *et al.*, 1982). The activity is associated with a fraction of the MT antigen having an apparent mol. wt. of ~200 000, compared with the MT monomer mol. wt. of 48 000 (Walter *et al.*, 1982). In principle, the protein kinase could be an intrinsic property of MT antigen, or else a cellular tyrosine protein kinase associated with the MT antigen. The failure to obtain protein kinase activity with MT antigen-related molecules synthesized in bacteria (B.Schaffhausen and T.Benjamin, personal communication; K.Palme and W.Eckhart, unpublished results) and a recent report indicating that pp60$^{c\text{-}src}$ is associated with MT antigen (Courtneidge and Smith, 1983) suggest that the latter alternative is most likely correct.

Polyoma virus expresses three T antigens, large, middle-sized and small. The MT antigen alone, however, is capable of transforming established cell lines (Treisman *et al.*, 1981). The protein kinase activity is closely correlated with the transforming ability of the MT antigen: most mutants which fail to transform lack the activity including the *hr*-t mutant NG-59 and the deletion mutant *dl*-23 (Eckhart *et al.*, 1979; Smith *et al.*, 1979; Schaffhausen and Benjamin, 1979, 1981), although there are exceptions such as *dl*-1015 (Schaffhausen and Benjamin, 1981) and *dl*-2208 (Nilsson *et al.*, 1983). Conversely all mutations, such as the termination mutants MOP 1033 (Templeton and Eckhart, 1982) and 1387-T (Carmichael *et al.*, 1982), which reduce the protein kinase activity also decrease transformation.

The site(s) of phosphorylation of the MT antigen *in vitro* has been investigated by analysis of partial proteolytic digestion products of wild-type and deletion mutant MT antigens (Schaffhausen and Benjamin, 1981). These experiments identified a major site of phosphorylation as tyrosine residue 315. This tyrosine residue is immediately downstream from an unusual run of six glutamic acid residues, and is in a region of the protein which shows some homology with the polypeptide hormone, gastrin (Baldwin, 1982).

We have used an alternative approach to identify phosphorylation sites in the MT antigen. This involves amino acid sequence analysis of peptides of the MT antigen derived by exhaustive tryptic digestion of protein containing radioactive phosphate groups introduced by *in vitro* phosphorylation. Because the amino acid sequence of the MT antigen is known, the site of phosphorylation can be deduced from a knowledge of the position of the phosphorylated residue in a particular tryptic peptide. This approach was used successfully to identify the single phosphorylated tyrosine residue in the Rous sarcoma virus transforming protein, pp60$^{v\text{-}src}$ (Patschinsky *et al.*, 1982). We describe here a similar analysis for MT antigen which reveals a second major site of *in vitro* phosphorylation, tyrosine residue 250. There are also other sites of phosphorylation whose accessibility may depend upon the antiserum used for immunoprecipitation.

Results

Tryptic peptide analysis of phosphorylated MT antigens

Figure 1 is a schematic representation of the MT antigen, showing the location of the tyrosine-containing tryptic peptides in the protein. The positions and extents of the deletion mutations, *dl*-8 (Smolar and Griffin, 1981) and *dl*-45 (Bendig *et al.*, 1980), are also indicated. To identify phosphorylation sites in MT antigen, we prepared tryptic digests from MT antigens phosphorylated *in vitro* in immunoprecipitates of extracts of mouse 3T6 cells infected by wild-type, *dl*-8 and *dl*-45 viruses made with either rat anti-tumor serum or IgG directed against the COOH-terminal hexapeptide of MT antigen (Walter *et al.*, 1981).

Figure 2 shows the phosphorylated tryptic peptides of a number of preparations of MT antigen, separated by electrophoresis at pH 8.9 followed by chromatography. In most cases the maps show a major phosphorylated basic peptide (peptides 1 and 1′), and to its acidic side two somewhat diffuse slightly off-vertical arrays of peptides (peptides 2, 2′, 3 and 3′). For the preparation shown in Figure 2K both the total digest and the purified peptides 1′, 2 and 3 contained only phosphotyrosine. In general, peptide maps of MT antigens phosphorylated in immunoprecipitates prepared with

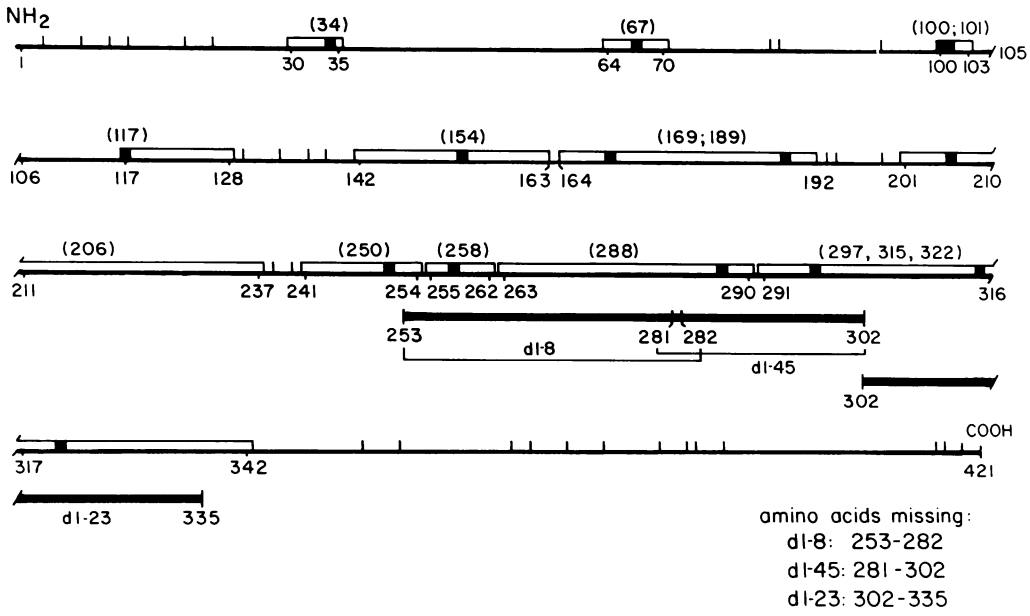

Fig. 1. A diagrammatic representation of the tryptic peptides of polyoma MT antigen. Polyoma MT antigen is presented as a horizontal line with short vertical lines representing the locations of all possible trypsin cleavage sites. The predicted amino acid sequence is based on the published nucleotide sequence of the polyoma virus early region (Friedmann *et al.*, 1979; Soeda *et al.*, 1979) and the published structure of the spliced MT antigen mRNA (Treisman *et al.*, 1981). Enclosed bars surround tryptic peptides containing tyrosine. The numbers under these bars correspond to the amino acid numbers of the NH_2- and COOH-terminal amino acids of the tryptic peptides. The numbers in brackets correspond to the amino acid numbers of the tyrosines contained in these peptides. The positions of the tyrosines within the peptides are indicated as black squares. The amino acids deleted from *dl*-8, *dl*-23 (Smolar and Griffin, 1981) and *dl*-45 (Bendig *et al.*, 1980) MT antigens are also given and the positions of the deletions shown as black bars.

anti-tumor serum (Figure 2D, E and F) are similar to those from MT antigen precipitated with anti-COOH peptide IgG (Figure 2A, B and K). We did find that the relative amounts of the major basic peptide and the acidic peptides varied from preparation to preparation and that occasionally there were new peptides, but this occurred regardless of the serum used to precipitate MT antigen (e.g., compare Figure 2A and K). Analysis of mixtures of digests showed that the major basic phosphopeptide (peptide 1) in the wild-type and *dl*-45 MT antigen digests co-migrated (Figure 2H). The major basic phosphopeptide (peptide 1′) in the *dl*-8 MT antigen digest, however, had a slightly different mobility in both dimensions (Figure 2C, G and I). This suggests that the *dl*-8 mutation results in an alteration in peptide 1, and we have therefore called the *dl*-8 peptide, peptide 1′ (although it is conceivable that peptide 1′ originates from a completely different site of phosphorylation in the *dl*-8 MT antigen, this seems unlikely). Peptides 2 and 3 co-migrated in a mixture of wild-type and *dl*-8 MT antigen digests (Figure 2G). In the *dl*-45 MT antigen digest, however, both sets of minor acidic peptides (peptides 2′ and 3′) had a greater chromatographic mobility than peptides 2 and 3 in the *dl*-8 and wild-type MT antigen digests (Figure 2H and I).

We had previously noted that immunoprecipitates made from *dl*-8-infected cells with the anti-COOH-peptide IgG exhibited a specific 30 000-dalton phosphoprotein, in addition to MT antigen, following *in vitro* phosphorylation (Walter *et al.*, 1981). The phosphopeptide map of this 30 000-dalton protein was identical to that of the intact *dl*-8 MT antigen (Figure 2K and L). Based on its recognition by the anti-COOH-peptide IgG it seems likely that the 30 000-dalton protein is a COOH-terminal fragment of MT antigen. Therefore all the major phosphorylation sites detectable by tryptic peptide mapping are located in the COOH-terminal half of MT antigen.

Phosphorylation of soluble MT antigen prepared by immunoaffinity binding and release from immobilized anti-COOH-peptide IgG is generally more efficient than phosphorylation in the bound state (Walter *et al.*, 1982; and unpublished observations). Examination of tryptic digests of MT antigen phosphorylated in solution, however, did not show consistently any new phosphopeptides while peptides 2 and 3 were under-represented (Figure 2J). The explanation for the greater efficiency of phosphorylation of MT antigen in solution may lie in the accessibility of the preferred phosphorylation sites in a greater fraction of the MT antigen molecules.

To characterize the structures of the tryptic phosphopeptides further, both the total tryptic digests and purified peptide 1′ were subjected to secondary digestion with *Staphylococcus aureus* protease V8 (specific for glutamic acids residues) followed by two-dimensional separation (data not shown). Neither peptide 1 nor 1′ showed an altered mobility after digestion, implying that both lack glutamic acid. In contrast, the acidic peptides were sensitive to digestion by *S. aureus* protease V8.

Sequence analysis of phosphopeptides

To define the location of the major basic phosphopeptide in MT antigen we subjected the purified peptide to automated sequence analysis in a spinning cup sequenator. Such analysis requires at least 3000 c.p.m. of purified peptide. This meant that we were able to analyze only the peptide from *dl*-8 MT antigen which is phosphorylated considerably more efficiently than wild-type or *dl*-45 MT antigens in immunoprecipitates. Figure 3 shows the amount of radioactivity released at each cycle during automated Edman degradation of peptide 1′. The phosphorylated residue was 10 residues from the NH_2 terminus of the peptide.

The only tyrosine residue in *dl*-8 MT antigen 10 residues to

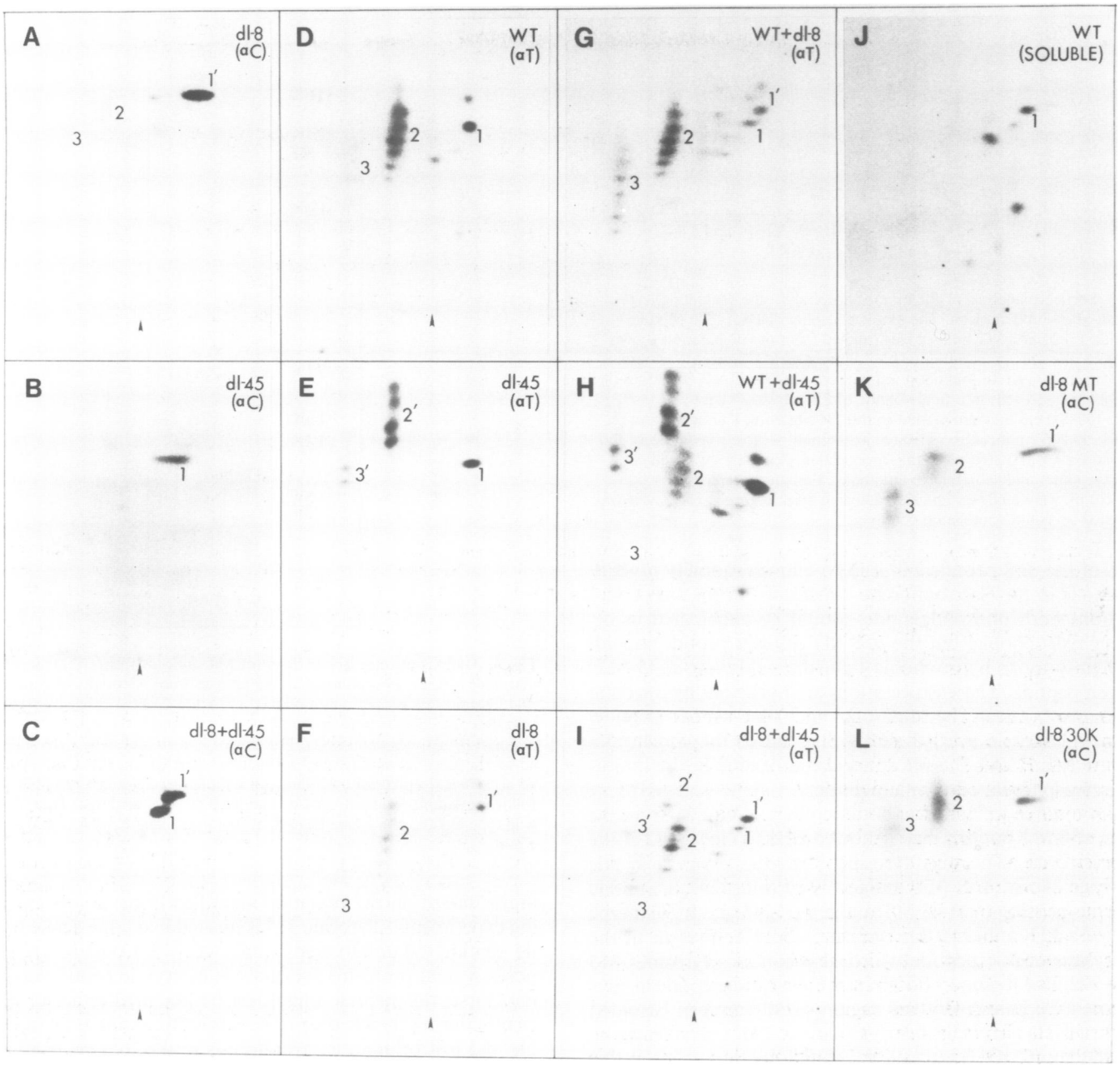

Fig. 2. The tryptic peptide patterns of phosphorylated MT antigen. Cultures of 3T6 cells were infected with polyoma large plaque wild-type virus and the viable deletion mutants *dl*-8 and *dl*-45 at a multiplicity of 10 p.f.u./cell. Routinely cell lysates were prepared 28 h post-infection from a 9 cm plate of infected cells, immunoprecipitated with either anti-tumor serum (αT) or anti-COOH-peptide (αC) IgG as indicated, and analyzed for protein kinase activity. The phosphorylated MT was isolated, subjected to trypsin digestion and the resultant tryptic peptides were separated on 100 u cellulose thin layer plates by electrophoresis at pH 8.9 with the anode on the left followed by ascending chromatography. The sample origin is shown by an arrowhead. The amounts of radioactivity and exposure times at −70°C to presensitized film with an intensifying screen are given. The following digests were analyzed: **A**, *dl*-8 MT antigen, anti-COOH-peptide IgG, 1125 c.p.m., 17 h; **B**, *dl*-45 MT antigen, anti-COOH-peptide IgG, 1200 c.p.m., 17 h ; **C**, mixture of *dl*-8 and *dl*-45 MT antigens, anti-COOH-peptide IgG, 1125 c.p.m. and 1200 c.p.m., respectively, 36 h; **D**, wild-type MT antigen, anti-tumor serum, 800 c.p.m., 7 days; **E**, *dl*-45 MT antigen, anti-tumor serum, 1000 c.p.m., 7 days; **F**, *dl*-8 MT antigen, anti-tumor serum, 1500 c.p.m., 4 days; **G**, mixture of wild-type and *dl*-8 MT antigens, anti-tumor serum, 200 c.p.m. and 270 c.p.m., respectively, 7 days; **H**, mixture of wild-type and *dl*-45 MT antigens, anti-tumor serum, 220 c.p.m. and 400 c.p.m., respectively, 7 days; **I**, mixture of *dl*-8 and *dl*-45 MT antigens, anti-tumor serum, 220 c.p.m. and 400 c.p.m., respectively, 7 days; **J**, wild-type MT antigen purified by binding to anti-COOH-peptide IgG linked to Sepharose followed by release with peptide and phosphorylation in solution, 500 c.p.m., 42 h; **K**, *dl*-8 MT antigen, anti-COOH-peptide IgG, 1600 c.p.m., 4 days; **L**, 30 000-dalton fragment of *dl*-8 MT antigen from the same immunoprecipitate as in **K**, 2400 c.p.m., 4 days.

the COOH-terminal side of a tryptic cleavage site is amino acid 250 (Figure 1). The deletion in *dl*-8 removes the COOH terminus of the tryptic peptide containing tyrosine 250 in wild-type MT antigen; the mutation in *dl*-45 does not affect this peptide. Thus in *dl*-8 MT antigen the peptide containing tyrosine 250 has the sequence Leu.Pro.Ser.Leu.Leu.Ser.Asn.Pro.Thr.Tyr.Ser.Val.Ser.Pro.Met.Thr.Ala.Tyr.Pro.Arg, while in wild-type and *dl*-45 MT antigens it has the sequence Leu.Pro.Ser.Leu.Leu.Ser.Asn.Pro.Thr.Tyr.Ser.Val.Met.Arg. The *dl*-8 MT antigen phosphopeptide (mol. wt. 2273) and the wild-type/*dl*-45 MT antigen phosphopeptide (mol. wt. 1657) will have the same charge (−2 at pH 8.9), but the greater

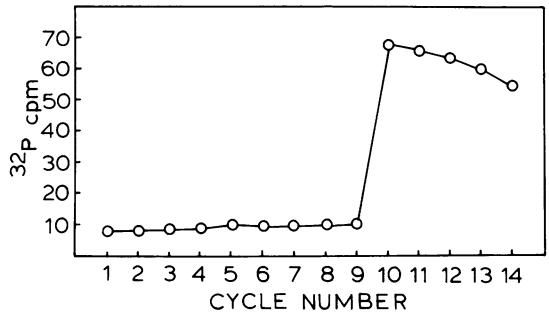

Fig. 3. Sequence analysis of the major phosphotyrosine-containing tryptic peptide of *dl*-8 MT antigen. The major phosphorylated tryptic peptide (peptide 1′) of *dl*-8 MT antigen was isolated from the preparation shown in Figure 2K. This preparation of phosphorylated MT antigen was obtained by incubation of an immunoprecipitate made from 12 x 9 cm dishes of *dl*-8-infected 3T6 cells in a final volume of 2.4 ml with 4.8 mCi of [γ-^{32}P]-ATP. 130 000 c.p.m. were present in the ^{32}P-labeled MT antigen band on the gel. 50 000 c.p.m. were recovered in the final soluble tryptic digest and resolved on 4 x 100 μ cellulose thin layer plates. Peptide 1′ was eluted from the plates and subjected to automated Edman degradation. 4000 c.p.m. were analyzed and the radioactivity emerging at each cycle was estimated by liquid scintillation counting.

mass of the *dl*-8 peptide will lead to a lower mobility toward the anode in the electrophoretic dimension. From their compositions both phosphopeptides would be anticipated to be rather hydrophobic, but the *dl*-8 MT antigen peptide should be slightly more hydrophobic than the wild-type/*dl*-45 MT antigen peptide. These expectations are borne out by the data in Figure 2. Given the additional fact that neither peptide contains glutamic acid, these results lead to the conclusion that tyrosine 250 is the phosphorylated residue in the major basic phosphorylated tryptic peptide.

Upon sequence analysis, both peptides 2′ and 3′ from the same *dl*-8 MT antigen digest showed peaks in low yield at the seventh cycle. The only tyrosine 7 residues from a tryptic cleavage site in *dl*-8 MT antigen is tyrosine 297, a residue which is missing in *dl*-45 MT antigen (Figure 1). In wild-type and *dl*-8 MT antigens this tyrosine would be present in the very large tryptic peptide which also contains tyrosines 315 and 322 (see below). Possibly these acidic peptide arrays represent peptides from this region of MT antigen bounded on their NH_2-terminal sides by threonine 291 with at least one set having COOH termini generated by non-trypsin like cleavages. The incremental separations of these peptides within each set in the chromatography dimension and the acid to basic trend of these peptide arrays going from bottom to top is suggestive of each set being multiple charge isomers of the same peptide. Part of the charge heterogeneity could be attributed to phosphorylation at multiple tyrosines, although there are certainly other explanations. The peptide from the equivalent region of *dl*-45 MT antigen, although distinct from that of wild-type and *dl*-8 MT antigens, would also be large (see below) and contain tyrosines 315 and 322. The fact that these acidic peptide arrays are different in the digests of *dl*-45 and wild-type/*dl*-8 MT antigens reinforces the notion that these peptides arise from this region of MT antigen. The low yield of radioactivity released at cycle 7 during sequencing could be accounted for if much of the phosphate were located on tyrosine 315.

Phosphorylation of tyrosine 315 in the MT antigen and in a synthetic peptide

Tyrosine 250, rather than tyrosine 315, appeared to be the

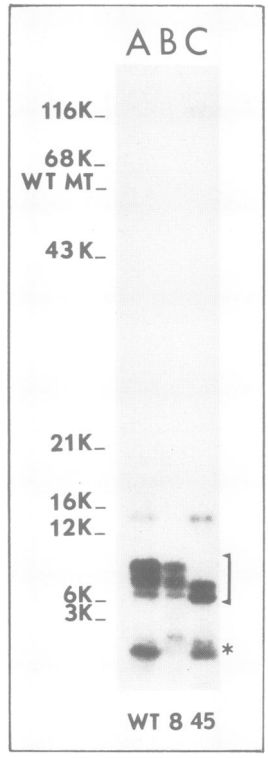

Fig. 4. Analysis of total tryptic digest of MT antigens from wild-type, *dl*-8 and *dl*-45 viruses. Phosphorylated MT antigen from wild-type, *dl*-8 and *dl*-45 viruses was prepared as described in Figure 2. After extensive trypsinization, the digests were analyzed by electrophoresis on a 20% acrylamide/0.07% bisacrylamide gel. Proteins of known mol. wts. were analyzed on the same gel and detected by staining. The gel was dried and exposed at −70°C to presensitized film with an intensifying screen for 18 h. The following samples and amounts of radioactivity were analyzed: **Lane A**, wild-type, 3000 c.p.m.; **lane B**, *dl*-8, 5000 c.p.m.; **lane C**, *dl*-45, 4000 c.p.m.

major site of phosphorylation identified in our tryptic mapping experiments. Tyrosine 315 is located in a large acidic tryptic peptide (52 amino acids, mol. wt. 6155) (Figure 1). This poses several problems for analysis by tryptic peptide mapping. First, this peptide might not be liberated intact or efficiently during tryptic digestion. Secondly, it might be insoluble and lost prior to electrophoresis in the step we routinely use to remove insoluble cores. Thirdly, we do not know if such a large peptide can be resolved by our two-dimensional technique. In addition, we estimated that the recovery of radioactivity in the major tryptic peptide from phosphorylated MT antigen was low. We were therefore prompted to try to detect the tryptic peptide containing the phosphorylated tyrosine 315 residue by an alternative method. To do this we analyzed total tryptic digests (without removal of insoluble material) of wild-type, *dl*-8 and *dl*-45 MT antigen, phosphorylated in immunoprecipitates made with rat anti-tumor serum, by electrophoresis on high percentage SDS-polyacrylamide gels. Figure 4 shows the result of such an analysis. A major triplet, of mol. wt. ~7000, was found in all three preparations (bracketed in Figure 4). The triplet in the *dl*-45 preparation migrated slightly faster than that in the wild-type and *dl*-8 preparations. The lowest band of the triplet may be an artefact of the gel system since the middle band generated material co-migrating with the lowest band upon re-running. All three bands were sensitive to digestion with *S. aureus* V8 protease although high concentrations were

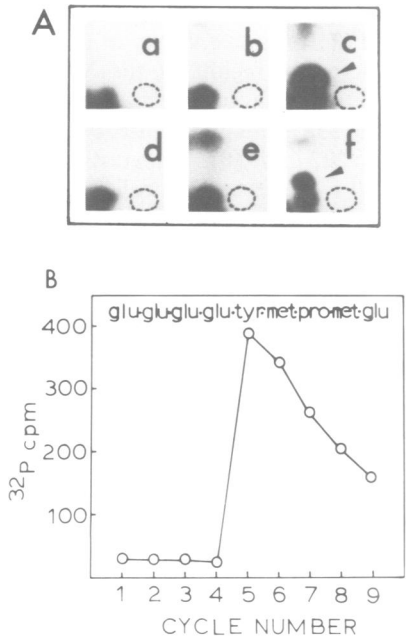

Fig. 5. Phosphorylation of the synthetic peptide Glu.Glu.Glu.Glu.Tyr.Met.Pro.Met.Glu. **(A)** Immunoprecipitates were prepared from either wild-type or mock-infected 3T6 cells 28 h post-infection with either anti-tumor serum **(panels a – c)** or anti-COOH-peptide IgG **(panels d – f).** The phosphorylation reactions were carried out as described in Materials and methods. The supernatants from these reactions were resolved by electrophoresis at pH 3.5 (horizontal dimension) followed by electrophoresis at pH 8.9 (vertical dimension). The plates were exposed at −70°C to presensitized film with an intensifying screen for 14 h. Because of the complexity of the pattern generated by the labeled ATP only a part of each autoradiogram is shown. The origin was to the lower center. The dashed circles represent the position of the unphosphorylated peptide. Arrowheads indicate the phosphorylated form of the peptide. The following reactions were analyzed: **a**, immunoprecipitate of *dl*-8-infected cells with anti-tumor serum; **b**, immunoprecipitate of mock-infected cells with anti-tumor serum and synthetic peptide; **c**, immunoprecipitate of *dl*-8-infected cells with anti-tumor serum and synthetic peptide; **d**, immunoprecipitate of *dl*-8-infected cells with anti-COOH-peptide IgG; **e**, immunoprecipitate of mock-infected cells with anti-COOH-peptide IgG and synthetic peptide; **f**, immunoprecipitate of *dl*-8-infected cells with anti-COOH-peptide IgG and synthetic peptide. **(B)** Phosphorylated synthetic peptide was isolated from a thin layer plate and subjected to automated Edman degradation as described previously. 10 000 c.p.m. were analyzed and the radioactivity emerging at each cycle was estimated by Cerenkov radiation.

required (data not shown).

Do these bands represent the tryptic peptide containing tyrosine 315? The estimated size of this triplet is close to that expected, and the mobilities of the bands are the same in the digests of wild-type and *dl*-8 MT antigens as anticipated. The deletion in *dl*-45 alters the peptide containing tyrosine 315, generating a slightly longer peptide (58 amino acids, mol. wt. 6852). On this basis, one would have predicted that the mobility of this *dl*-45 peptide should be lower than that of the wild-type/*dl*-8 peptide. It is known, however, that this region of MT with its high proline and acidic amino acid content contributes to the mol. wt. anomaly observed for MT antigen. It seems likely therefore that this triplet represents the tryptic peptide containing tyrosine 315.

A faster migrating band was observed in all three digests with a mol. wt. estimated to be between 1500 and 2500 (indicated by an asterisk in Figure 4). This phosphopeptide in the *dl*-8 MT antigen digest migrated slightly more slowly than the equivalent peptides in the wild-type and *dl*-45 MT antigen digests suggesting it might in fact be the peptide containing tyrosine 250. This peptide was 2 – 3 times less abundant than the combined members of the triplet.

We sought further evidence that tyrosine 315 could be phosphorylated by the MT antigen-associated protein kinase by adding the synthetic peptide, Glu.Glu.Glu.Glu.Tyr.Met.Pro.Met.Glu, corresponding to the amino acid sequence of MT antigen around tyrosine 315, to immunoprecipitates containing MT antigen. We had previously found that this peptide could be phosphorylated by immunoprecipitates containing the Y73 virus transforming protein P90$^{gag\text{-}yes}$ with its associated tyrosine protein kinase activity (Hunter, 1982). Immunoprecipitates containing *dl*-8 MT antigen precipitated with either anti-tumor serum or anti-COOH-peptide IgG were able to phosphorylate the synthetic peptide (Figure 5A). Immunoprecipitates made from uninfected cells with either serum or from *dl*-8-infected cells with normal serum possess no peptide phosphorylating activity (Figure 5A). The isolated phosphopeptide contained only phosphotyrosine and, as expected, was sensitive to digestion with *S. aureus* protease V8 (data not shown). The identity of the phosphorylated product was confirmed by automated sequence analysis which showed the phosphorylated residue to be at position 5 as anticipated (Figure 5B). The K_m for peptide in the phosphorylation reaction was ~3 mM. This value is similar to that observed for this peptide with P90$^{gag\text{-}yes}$. These results show that the MT antigen-associated protein kinase can phosphorylate an exogenously added synthetic peptide containing a tyrosine residue with a surrounding amino acid sequence like that of tyrosine 315.

Discussion

We have shown here that the polyoma MT antigen-associated protein kinase phosphorylates at least two of the 15 tyrosine residues in MT antigen in immunoprecipitates, namely tyrosines 250 and 315. Although tyrosine 315 was originally identified as the major phosphorylated residue (Schaffhausen and Benjamin, 1981), recent observations have indicated that other sites of phosphorylation are likely to be present. If tyrosine 315 is converted to phenylalanine by site-specific mutagenesis, tyrosine phosphorylation of the MT antigen lacking tyrosine 315 is still observed (Oostra *et al.*, 1983; G.Carmichael, personal communication). In addition, when tyrosines 315 and 322 are sequestered by binding specific anti-peptide antibodies, other tyrosine residues in MT antigen are phosphorylated (A.Smith, personal communication). These observations taken together with our results provide compelling evidence for the existence of multiple tyrosine phosphorylation sites in the COOH-terminal half of MT antigen.

Why was phosphorylation of tyrosine 250 not observed previously? Several factors can affect the patterns of MT antigen phosphorylation, including the precise conditions used for growth and infection of cells and for preparation of immunoprecipitates. Different types of anti-tumor serum clearly behave differently; for example, some antisera allow phosphorylation of immunoglobulin molecules in the immunoprecipitates, while others do not (Smith *et al.*, 1979). We have also observed some variability in the relative intensities of tryptic phosphopeptides with individual anti-tumor sera and the anti-COOH-peptide IgG. Possibly there are antibodies in some polyclonal anti-tumor sera which block potential sites of phosphorylation. In this respect it is of interest that Schaffhausen *et al.* (1982) observed a drastic reduction in MT

antigen-associated kinase activity when immunoprecipitates were prepared with an antiserum directed against a synthetic peptide corresponding to residues 311–319, whereas other studies with similar anti-peptide sera showed nearly normal levels of MT antigen-associated kinase activity (A.Smith; Y.Ito, personal communications).

Although we have identified at least one other site of tyrosine phosphorylation in MT antigen, our gel analysis of tryptic digests of phosphorylated MT antigen suggests that the tryptic peptide comprising residues 291–342 contains the bulk of the phosphate. Since tyrosine 315 is in this peptide it could be the major phosphorylation site in MT antigen as suggested by Schaffhausen and Benjamin (1981). Tyrosines 297 and 322, however, are also in this peptide and our results do not distinguish between phosphorylation at tyrosine 315 and at the other two tyrosines. Indeed, one of the minor phosphorylation sites we have observed may be tyrosine 297, while Alan Smith and colleagues have found that tyrosine 322 can be phosphorylated under certain conditions (personal communication). The fact that the synthetic peptide Glu.Glu.Glu. Glu.Tyr.Met.Pro.Met.Glu was phosphorylated by MT antigen-containing immunoprecipitates corroborates identification of tyrosine 315 as a phosphate acceptor in intact MT antigen. In this context it is noteworthy that gastrin, which contains a tyrosine embedded in a homologous sequence, can be phosphorylated by the epidermal growth factor-stimulated tyrosine protein kinase in A431 cell membranes (Baldwin *et al.*, 1982, 1983).

Recent evidence suggests that the phosphotransferase activity associated with MT antigen may not be intrinsic (Courtneidge and Smith, 1983). Because MT antigen can associate with $pp60^{c\text{-}src}$ and potentially with other cellular tyrosine protein kinases, the nature of the putative kinase binding site on MT antigen is of obvious interest. The fact that *dl*-23 MT antigen, which lacks residues 301–335, has a low associated tyrosine kinase activity was originally interpreted to mean that the acceptor site was missing (Schaffhausen and Benjamin, 1981). Given the results of replacing tyrosine 315 and the observation that tyrosine 315 is not the sole phosphorylation site, there must be another explanation for the decreased kinase activity of *dl*-23 MT antigen. Possibly the kinase might bind to this highly acidic part of MT antigen, although there are other deletion mutations in this region whose MT antigens have high levels of associated protein kinase activity (Schaffhausen and Benjamin, 1981; Mes and Hassell, 1982; Nilsson *et al.*, 1983). Furthermore, the *hr*-t mutant NG-59 MT antigen (Eckhart *et al.*, 1979; Schaffhaussen and Benjamin, 1979), bearing a single amino acid substitution and insertion at position 179, and a series of NH_2-terminal deletion mutants also lack protein kinase activity (D.Templeton, unpublished observations), potentially implicating both these regions in recognition by the kinase as well. Wherever the kinase binding site is located it is notable that all the acceptor tyrosines are in the COOH-terminal half of MT antigen.

Is phosphorylation of tyrosine important for the function of the MT antigen? Although the MT antigen isolated from infected cells is not highly phosphorylated and contains mainly phosphoserine with a trace of phosphotyrosine (Eckhart *et al.*, 1979; Schaffhausen and Benjamin, 1979, 1981; Segawa and Ito, 1982), a subpopulation of the MT antigen, phosphorylated on tyrosine, might be an important functional species. Such a population could not be studied easily using current biochemical methods and reagents. Mutagenesis of the tyrosine residues which are potential sites of phosphorylation may be more informative. Many of the tyrosine residues in the COOH-terminal region of the MT antigen can be removed by deletion without affecting transformation. For example, tyrosine 258 is missing in *dl*-8, tyrosines 289 and 297 in *dl*-45, tyrosine 322 in *dl*-1013 (Magnusson *et al.*, 1981) and tyrosine 315 in *dl*-1-4 (Mes and Hassell, 1982). Conversion of tyrosine 315 to phenylalanine may reduce but does not abolish the ability of MT antigen to transform (Oostra *et al.*, 1983; G.Carmichael, personal communication). The effect of conversion of tyrosine 250 to phenylalanine on the transforming potential of MT antigen is currently being assessed by Alan Smith and his colleagues.

Materials and methods

Cell culture, virus infection and lysate preparation

The preparation of polyoma-infected cell lysates has been described (Hutchinson *et al.*, 1978). Briefly, mouse 3T6 cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 5% calf serum at 37°C to a density of 2 x 10^6/9 cm dish. The cells were infected at a multiplicity of ~20 p.f.u. with plaque stocks of wild-type polyoma or various mutants, as indicated. At 28 h post-infection, each plate of infected cells was lysed in 1 ml of extraction buffer: 0.15 M NaCl, 1% sodium deoxycholate, 1% Nonidet P40, 0.1% SDS, 10 mM sodium phosphate, pH 7.0, 1% Trasylol. The resulting lysate was clarified at 27 000 *g* for 30 min. Clarified lysates were routinely stored in liquid nitrogen.

Immunoprecipitation and protein kinase assay

Immunoprecipitation with polyoma anti-tumor serum and an antiserum against a hexapeptide corresponding to the COOH terminus of MT antigen was performed as described previously (Hutchinson *et al.*, 1978; Walter *et al.*, 1981). Partial purification of the MT antigen using immunoaffinity chromatography was performed as described (Walter *et al.*, 1982) except that the anti-COOH-peptide IgG was cross-linked to protein A Sepharose using dimethyl pimelimidate instead of CNBr-activated Sepharose (Schneider *et al.*, 1982), a procedure which results in less inactivation of the antibody. Protein kinase assays were carried out in immunoprecipitates (Eckhart *et al.*, 1979) except that in some cases the buffer used was extraction buffer supplemented with 10 mM $MgCl_2$ and 20 μCi [γ-^{32}P]ATP/50 μl reaction or in solution after release of MT antigen bound to antibody-Sepharose conjugates (Walter *et al.*, 1982). Phosphorylated MT antigen was isolated by SDS-polyacrylamide gel electrophoresis using 15% acrylamide cross-linked with 0.09% methylene bisacrylamide.

Tryptic peptide analysis

Phosphorylated MT antigen was extracted from the gel and tryptic peptides were prepared and analyzed by two-dimensional electrophoresis and chromatography as described (Hunter and Sefton, 1980) using 1% ammonium carbonate pH 8.9 as the electrophoresis buffer and a chromatography buffer composed of n-butanol:pyridine:acetic acid:water at ratios of 375:250:75:300. Size estimation of the larger phosphopeptides was carried out using SDS-polyacrylamide electrophoresis on 20% acrylamide/0.07% bisacrylamide gel using glucagon, bovine trypsin inhibitor, cytochrome c, myoglobin, soya bean trypsin inhibitor, ovalbumin, bovine serum albumin and β-galactosidase as size standards. Sequence analysis of the phosphorylated tryptic peptides was performed by automated Edman degradation as described (Patschinsky *et al.*, 1982).

Phosphorylation of synthetic peptide Glu.Glu.Glu.Glu.Tyr.Met.Pro.Met.Glu

The peptide was phosphorylated under the conditions previously described for 10 min at 30°C (Hunter, 1982). Each reaction contained 5 μl of an immunoprecipitate absorbed to *S. aureus* suspended in 20 mM 1,4-piperazinediethanesulfonic acid pH 7.0, 10 mM $MnCl_2$ (corresponding to 3 x 10^4 cells made as described above with the indicated antiserum), 1 μl of a 20 mM solution of peptide or H_2O and 1 μl of 40 μM [γ-^{32}P]ATP (150 μCi/nmol). After incubation, the bacteria were pelleted and 1 μl of the supernatant was resolved on 100 u cellulose thin layer plates by electrophoresis at pH 3.5 for 35 min at 1 kV followed by electrophoresis at right angles at pH 8.9 for 12 min at 1 kV. The peptide was detected by ninhydrin staining.

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