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# Phosphorylation of polyoma middle T antigen and cellular proteins in purified plasma membranes of polyoma virus-infected cells

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**We have studied phosphorylation carried out by purified plasma membranes from polyoma virus-infected cells. When isolated membranes are incubated with [$\gamma$-$^{32}$P]ATP, polyoma virus middle T antigen (mT) becomes phosphorylated on tyrosine. Partial proteolysis mapping shows the same pattern as previously noted for mT labeled in immune complexes. Membranes labeled *in vitro* were also extracted and immunoprecipitated with anti-T or anti-src antibody. With either antibody, both mT and pp60$^{c\text{-}src}$ were brought down and shown to be labeled on tyrosine. The mT of an *hr-t* mutant (NG59) showed only a trace amount of labeling in membranes under the same conditions. Proteins from infected and uninfected cell membranes labeled *in vitro* were separated on two-dimensional gels. An acidic 40-kd phosphoprotein was labeled in uninfected cell membranes, but was not seen using membranes from wild-type virus-infected cells. Neither NG59, which encodes a defective but membrane-associated mT, nor a mutant encoding a truncated mT that fails to associate with membranes, alters the level of the 40-kd phosphoprotein in membranes labeled *in vitro*. These results suggest that mT, acting through pp60$^{c\text{-}src}$ and possibly other cellular kinases and phosphatases, can affect cell protein phosphorylation as part of the transformation process.**

*Key words:* membrane proteins/phosphorylation/polyoma virus/pp60$^{c\text{-}src}$/middle T antigen

## Introduction

Polyoma virus encodes in the early region of its genome two genetically-defined functions that are crucial to the process of cell transformation (Eckhart, 1977; Fluck *et al.*, 1977). One of these genes, designated '*hr-t*', is responsible for most of the phenotypic alterations in transformed cells (Benjamin, 1982); it encodes two overlapping proteins, the small- and middle-sized T antigens, with mol. wts. of 22 kd and 56 kd, respectively. Middle T (mT) antigen has been shown to be phosphorylated *in vivo* on serine and/or threonine (Schaffhausen and Benjamin, 1979, 1981a; Segawa and Ito, 1982), and in an *in vitro* immunoprecipitate assay on tyrosine residues (Eckhart *et al.*, 1979). *Hr-t* and other mutants which fail to express an intact mT capable of being phosphorylated in the *in-vitro* kinase assay are defective in transformation (Benjamin, 1982; Carmichael *et al.*, 1982; Carmichael *et al.*, 1984; Schaffhausen and Benjamin, 1979; Smith *et al.*, 1979; Templeton and Eckhart, 1981), and the expression of mT antigen in established rat cells is sufficient to induce a transformed phenotype (Treisman *et al.*, 1981).

Middle T antigen molecules that are active in the *in-vitro* kinase reaction are localized in the plasma membrane (Schaffhausen *et al.*, 1982; Segawa and Ito, 1982). Truncated mTs that lack a hydrophobic carboxyterminal segment are defective in the kinase assay as well as in transformation (Carmichael *et al.*, 1982; Templeton and Eckhart, 1981). It is therefore assumed that cell transformation by polyoma is directly related to the viral ability to express an intact mT that is anchored in the plasma membrane. Several retroviral oncogene products are located in the plasma membrane and show intrinsic tyrosine-kinase activities (Hunter *et al.*, 1981). Tyrosine phosphorylation of mT in immune complexes seems not to be an intrinsic activity of the molecule (Schaffhausen *et al.*, 1982; Schaffhausen *et al.*, 1985) but rather a consequence of an interaction with pp60$^{c\text{-}src}$ (Courtneidge and Smith, 1983, 1984) also shown to reside in the plasma membrane (Courtneidge *et al.*, 1980; Courtneidge and Bishop, 1982). Recent evidence has shown that mT increases the kinase activity of pp60$^{c\text{-}src}$ in complexes of these two proteins (Bolen *et al.*, 1984). These observations give rise to the hypothesis that mT may act as a regulatory subunit to redirect or alter the activities of pp60$^{c\text{-}src}$ *in vivo* (Courtneidge and Smith, 1984).

To explore this hypothesis further, we have studied and compared phosphorylation in purified plasma membranes from polyoma virus-infected and uninfected cells. We report that mT is phosphorylated primarily on tyrosine when labeled in plasma membranes, and gives a partial proteolysis map identical to mT labeled in immune complexes (Schaffhausen and Benjamin, 1981a). Middle T constitutes a major species and pp60$^{c\text{-}src}$ a minor species among plasma membrane proteins phosphorylated on tyrosine *in vitro*. Serine and threonine modifications predominate in plasma membrane-associated cellular proteins. The appearance of one such protein is greatly reduced in membranes containing a functional mT protein.

## Results

### *Middle T antigen is phosphorylated in isolated membranes*

When purified plasma membranes of 3T3 cells infected with polyoma for 24 h are labeled with [$\gamma$-$^{32}$P]ATP, a multitude of cellular proteins are phosphorylated. Figure 1A shows the pattern of total phosphorylated membrane-associated proteins run on an SDS gel. No difference in the band pattern between membranes of cells infected with the various virus mutants is observed. When an SDS gel run in parallel is treated with NaOH after fixation, and subsequently dried and exposed to film, the pattern shown in Figure 1B is observed. This treatment preferentially removes serine- and threonine-bound phosphate, while preserving the label in proteins phosphorylated on tyrosine (Cooper and Hunter, 1981). Relatively few bands are alkali-stable, one of which is mT. The other bands of cellular origin are present in all membranes, including those derived from cells infected with transforming or non-transforming viruses or from mock-infected cells. B2 is an *hr-t* mutant with a frame-shift deletion (Hattori *et al.*, 1979) which does not express mT (Silver *et al.*, 1978). NG59 expresses a membrane-associated mT with an insertion/point mutation (Carmichael and Benjamin, 1980;

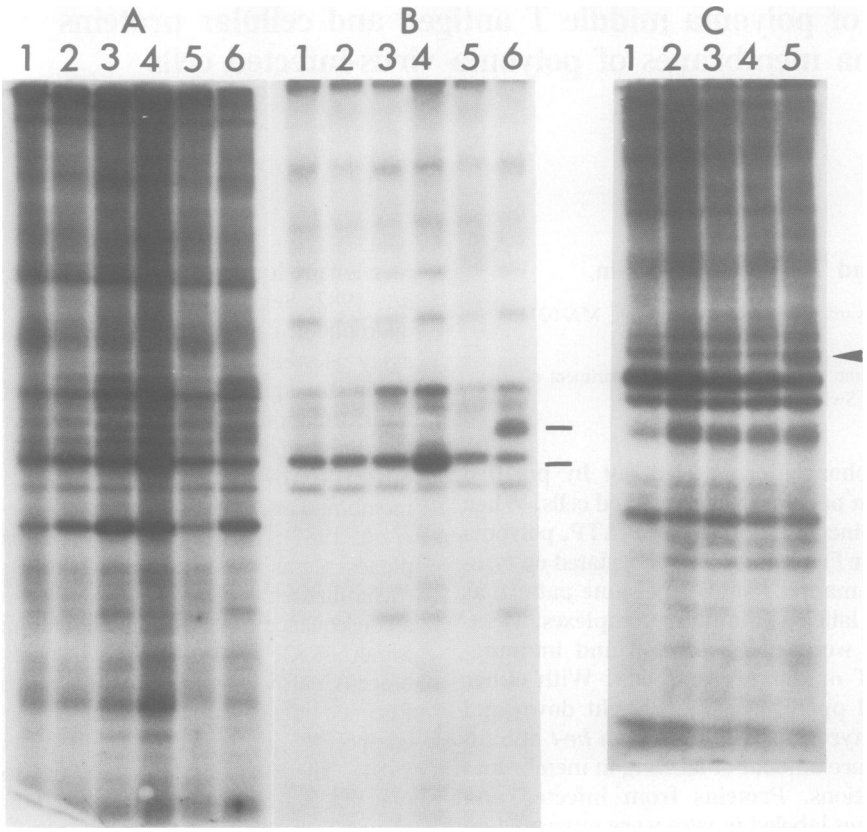


**Fig. 1.** Phosphorylation of purified plasma membranes from uninfected and virus-infected 3T3 cells. (**A**) Total phosphoproteins labeled in purified membranes. (**B**) Alkali-stable phosphoproteins labeled in purified membranes. (**C**) Alkali-stable phosphoproteins labeled in crude membranes. **A** and **B**, lanes are: (**1**) uninfected cells; (**2**) B2 infected cells; (**3**) NG59 infected cells; (**4**) dl45 infected cells; (**5**) 1387T infected cells; and (**6**) NG59RA infected cells. One fourth of the sample was used in A (exposure time was one day, without intensifier screen), and three fourths in B (exposure was for two days, with screen). **C**, lanes are: (**1**) uninfected cells; (**2**) B2 infected cells; (**3**) NG59 infected cells; (**4**) dl45 infected cells; and (**5**) NG59RA infected cells. The gel was alkali-treated and exposed for two days with a screen. Horizontal indicators in **B** show positions of wild-type (upper) and dl45 (lower) mT. Arrowhead in **C** shows position for wild type mT.

Silver *et al.*, 1978). The mutant 1387T expresses a soluble truncated form of mT lacking a C-terminal hydrophobic sequence required for membrane association (Carmichael *et al.*, 1982). All three mutants are transformation-defective and show no mT-associated kinase activity in the standard *in-vitro* (immune complex) kinase assay. No phosphorylated mT is seen in purified plasma membranes of cells infected with any one of these mutants. dl45 and NG59 RA are 'wild-type' virus strains with normal transforming ability and *in-vitro* kinase activity. dl45 expresses a shortened mT of mol. wt. 54 kd, while NG59RA shows a normal pattern in the *in-vitro* assay (Schaffhausen and Benjamin, 1981a). Phosphorylation of isolated membranes shows the expected mT species for these 'wild-type' strains (Figure 1B, lanes 4 and 6). Purified membranes from polyoma-transformed 3T3 cells also show labeling of mT in alkali-treated gels (not shown).

Incubations of membranes with [$\gamma$-$^{32}$P]ATP were carried out for different times, from 20 s to 20 min. Maximal incorporation was seen between three and five minutes. Longer incubations showed lower levels of incorporation as well as depletion of the ATP. Based on levels of incorporation into mT after a four minute incubation, the recovery of mT in the membrane preparations is in the same order of magnitude as that seen in immune complexes prepared directly from detergent-disrupted cells.

When crude membranes, i.e., a 40 000 *g* postnuclear pellet of hypotonically-shocked cells, are used for this assay, mT is only barely detected. Most of the alkali-stable phosphoproteins are of cellular origin, and mT is a very minor band in a much more complex pattern (see Figure 1C). mT of the deletion-mutant dl45 is barely visible because it overlaps with a band of cellular origin (Figure 1C, lane 4). The crude membranes contain a lot of material not originating from the plasma membrane, including loosely-bound extrinsic membrane proteins that are lost in the fractionation procedure used to make purified plasma membranes. A significantly higher background of alkali-stable phosphoproteins is therefore expected.

### *Middle T antigen and pp60$^{c\text{-}src}$ are labeled in isolated membranes and can be precipitated with either anti-polyoma or anti-src antiserum*

Figure 2A shows immunoprecipitates of mT labeled in isolated membranes using either monoclonal antibody 327 (Lipsich *et al.*, 1983) reactive with pp60$^{c\text{-}src}$ (lanes 1, 2, 3) or anti-polyoma T serum (lanes 4, 5, 6). The membranes are first incubated with [$\gamma$-$^{32}$P]ATP and then extracted with a buffer containing 1% NP40, 100 $\mu$m sodium vanadate and 10 mM NaF in an isotonic salt solution. The labeling patterns are the same with either antibody. 'Wild-type' strains dl45 (lanes 1 and 4) and NG59RA (lanes 3 and 6) show incorporation into their mT proteins at ~54 kd and 56 kd, respectively, while the non-transforming mutant NG59 does not. Weak labeling of a 60-kd band correspon-

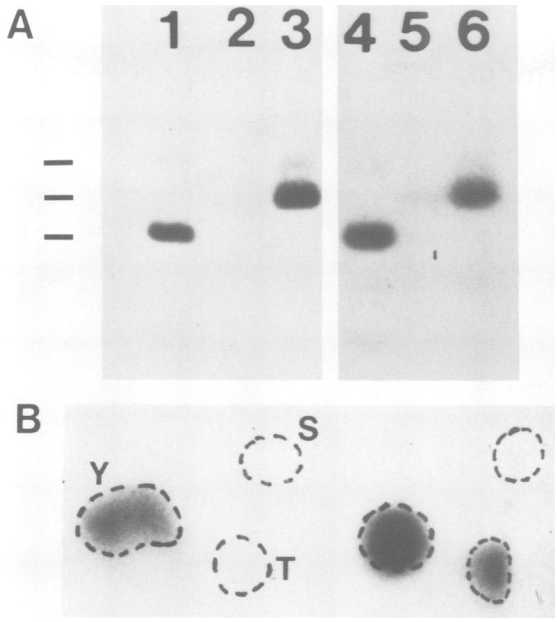


**Fig. 2.** (**A**) Immunoprecipitation of mT and pp60$^{c\text{-}src}$ proteins labeled in membranes. (**Lanes 1–3**): precipitated with anti-src monoclonal antibody; (**lanes 4–6**): precipitated with anti-polyoma serum; (**lanes 1** and **4**): dl45-infected cells (**lanes 2** and **5**): NG59-infected cells; (**lanes 3** and **6**): NG59RA-infected cells. Horizontal indicator shows positions of pp60$^{c\text{-}src}$ (upper), wild-type mT (middle), and dl45 mT (lower). Gel was exposed for four days with a screen. (**B**) Phosphoamino-acid analysis of pp60$^{c\text{-}src}$ and mT. The bands corresponding to pp60$^{c\text{-}src}$ and mT from Figure 1A (lane 1) were analyzed as described in Materials and methods. The direction of electrophoresis was vertical for the first dimension, from left to right for the second. Exposure of the thin layer plate was for two weeks with a screen. **Left panel**: phosphoamino acids from pp60$^{c\text{-}src}$; **right panel**: phosphoamino acids from mt.

ding to the size of pp60$^{c\text{-}src}$ is seen, only in membranes from 'wild-type' virus infected cells (lanes 1, 3, 4 and 6). These results of labeling isolated membranes confirm and extend those of Courtneidge and Smith who first demonstrated co-precipitation of mT and pp60$^{c\text{-}src}$ from whole-cell extracts (Courtneidge and Smith, 1983, 1984). Middle T-pp60$^{c\text{-}src}$ complexes show enhanced kinase activity toward exogenous substrates (Bolen *et al.*, 1984), and also increased autophosphorylation by pp60$^{c\text{-}src}$ involving tyrosines in the N-terminal part of the molecule (Yonemoto *et al.*, 1985). The results of Figure 2A are consistent with enhanced autophosphorylation of pp60$^{c\text{-}src}$ in isolated membranes that contain functional mT, or simply with a relative loss of pp60$^{c\text{-}src}$ from membranes lacking functional mT (NG59, lanes 2 and 5). Weak labeling of NG59 mT (lane 5) can be seen in longer exposures, indicative of a weak interaction with pp60$^{c\text{-}src}$ as recently shown (Bolen and Israel, 1985). Protein phosphatases, as well as ATPases, are clearly present and active in these membranes, since without fluoride and vanadate in the extraction buffer, little or no labeling of mT is observed.

*Protease mapping confirms phosphorylation of mT and pp60$^{c\text{-}src}$ proteins in plasma membranes*

Figure 3 shows protease V8 maps of alkali-stable phosphoproteins from plasma membranes labeled *in vitro*. Two fragments at 24 kd and 18 kd represent the major V8 C-terminal phosphotyrosine-containing fragments of wild-type mT as described earlier for mT labeled in immune complexes; dl45 shows a normal 18-kd fragment but the 24-kd fragment is shorter (Schaffhausen and Benjamin, 1981a). These results with NG59RA and dl45 are confirmed here (Figure 3, panels A and C). NG59, as expected, shows little or no labeling of these peptides. An alkali-stable V8 phosphopeptide of 26 kd is seen at the position of a protein with apparent mol. wt. of 60 kd (noted with an asterisk in Figure 3, panels A and B). This peptide is seen using either anti-src or anit-polyoma T antibody to precipitate material from membranes labeled *in vitro* (Figure 3, panel B). We suggest that this fragment represents the carboxyterminal portion of pp60$^{c\text{-}src}$, resulting from autophosphorylation in intact plasma membranes, as previously observed in immunoprecipitates (Collett *et al.*, 1979; Yonemoto *et al.*, 1985).

Phosphoamino-acid analyses on mT and pp60$^{c\text{-}src}$ bands cut out from non-alkali-treated one-dimensional gels were carried out by two-dimensional electrophoresis (Cooper and Hunter, 1981). The results show phosphotyrosine to be the major component (Figure 2B). Small amounts of phosphothreonine and phosphoserine were occasionally seen in mT.

*Changes in the phosphoprotein pattern in plasma membranes as detected in 2D gels*

When total proteins from plasma membranes phosphorylated *in vitro* are analyzed on two-dimensional gels, some discrete and reproducible differences are seen that depend on the presence of a functional mT in the membranes. Figure 4 shows two-dimensional gels of labeled membrane preparations from uninfected, *hr-t* mutant and wild-type infected cells. A phosphoprotein with an isoelectric point of approximately five and an apparent mol. wt. of 40 kd is present in both uninfected and mutant-infected cell membranes, but not in membranes from wild-type virus-infected cells. The mutant 1387-T, encoding a truncated cytoplasmic form of mT, also failed to alter the level of *in-vitro* phosphorylation of this phosphoprotein. The same behavior was seen using non-equilibrium two-dimensional gels separating basic as well as acidic proteins (not shown). This protein is phosphorylated predominantly on serine and/or threonine in membranes of uninfected or mutant-infected cells as judged by alkali lability, although a low level of phosphotyrosine has not been ruled out. Other but relatively minor quantitative differences correlating with the presence of functional mT protein may be seen in the two-dimensional gel patterns of *in-vitro* phosphorylated membrane proteins. These changes, particularly in the mol. wt. range below 30 kd, are not well reproducible. Using crude rather than purified membranes from wild-type virus-infected cells, the two-dimensional gel patterns are too complex to discern the absence of the 40-kd protein.

## Discussion

The mT protein of polyoma virus is a substrate for several protein kinases as shown by reactions carried out on immune complexes and by labeling infected cells with [$^{32}$P]orthophosphate. In the former case, mT becomes phosphorylated on tyrosine (Eckhart *et al.*, 1979), and in the latter case on serine and/or threonine at two distinct sites (Schaffhausen and Benjamin, 1979, 1981a; Segawa and Ito, 1982). The mT protein itself shows no ATP-binding (Schaffhausen *et al.*, 1982) or autokinase activity (Schaffhausen *et al.*, 1985). The tyrosine-kinase activity measured in immune complexes appears to reside in pp60$^{c\text{-}src}$ which co-precipitates with mT (Bolen *et al.*, 1984; Courtneidge and Smith, 1983, 1984; Yonemoto *et al.*, 1985). The serine/threonine kinases which phosphorylate mT *in vivo* are unknown, although recent evidence has indicated a role for protein kinase C in phosphorylation of the 58 kd form of mT (J.Matthews, T.Benjamin, in preparation).

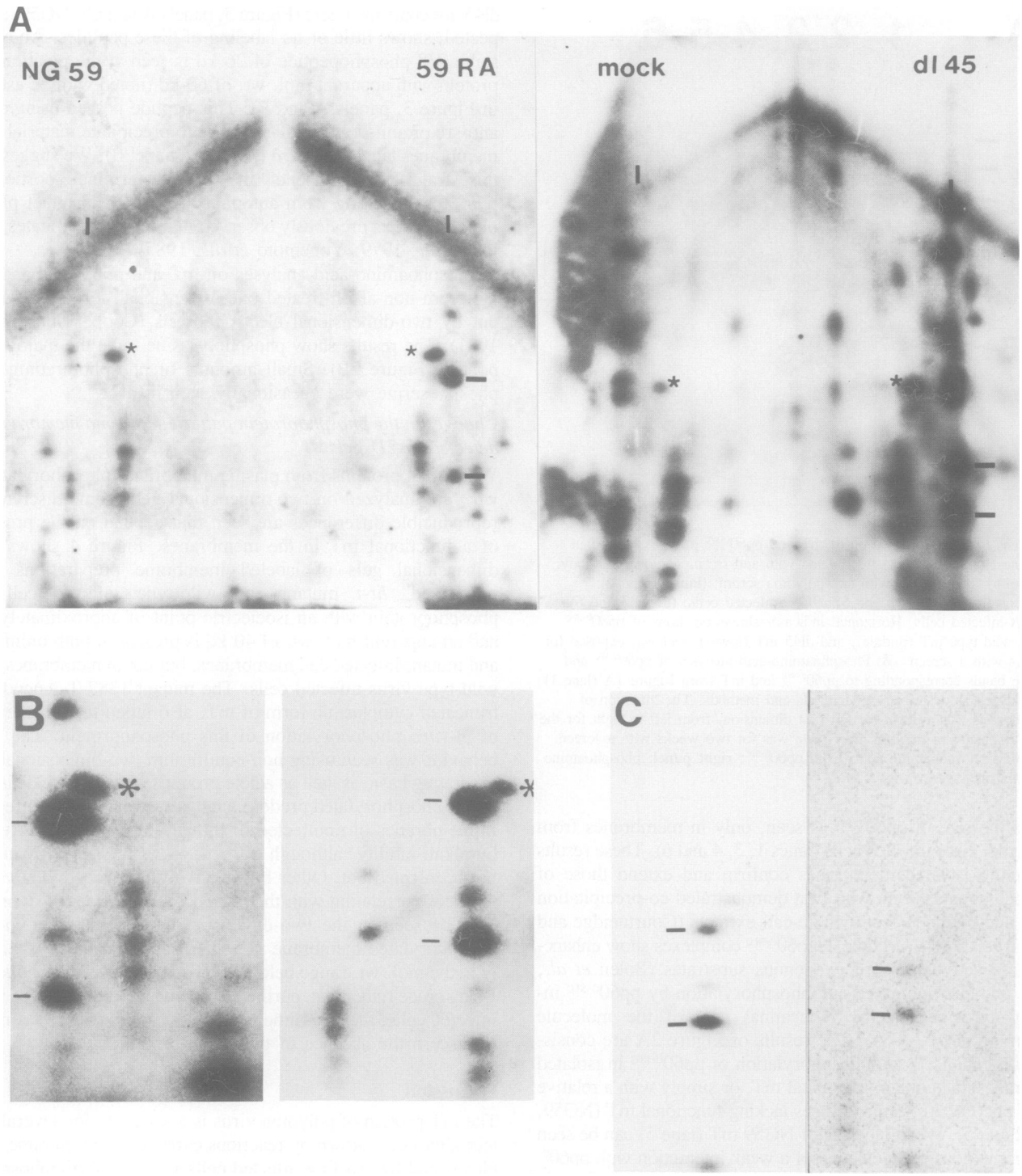


**Fig. 3.** V8 protease mapping of mT and pp60$^{c\text{-}src}$ labeled in plasma membranes. (**A**) Total plasma membranes labeled with [$\gamma$-$^{32}$P]ATP. The gels were alkali-treated after fixation. The vertical bar represents the position of mT in the first dimension. The horizontal bars represent the positions of the 24-kd (shortened in dl45) and 18-kd V8 fragments of mT. The asterisk indicates the position of the 26-kd carboxyterminal V8 fragment of pp60$^{c\text{-}src}$. Exposure was six days with a screen. (**B**) V8 digests of immunoprecipitates of membrane material from NG59RA infected cells labeled with [$\gamma$-$^{32}$P]ATP. **Left panel**: material was precipitated with anti-polyoma serum. **Right panel**: material was precipitated with anti-src serum. The immunoprecipitates were run and digested on separate gels and exposed (without alkali treatment) for eight days with a screen. The 24-kd and 18-kd fragment derived from mT are indicated by the horizontal bars. The asterisk indicates the 26-kd pp60$^{c\text{-}src}$-derived V8 fragment. (**C**) V8 digests of anti-polyoma immunoprecipitates from labeled membranes. **Left panel**: NG59RA-infected cell membranes, showing 18-kd and 24-kd cleavage products. **Right panel**: dl45-infected cell membranes, showing 18-kd and deleted 24-kd cleavage products. Exposure was eight days for NG59RA and 14 days for dl45, with screens.

Studies of virus mutants point clearly to the importance of mT phosphorylation in virus growth as well as cell transformation. Nevertheless, much remains obscure about the significance of each of the mT modifications. For example, despite the correlation between expression of the mT-associated tyrosine-specific *in-vitro* kinase activity and the viral ability to transform, there is little or no detectable phosphorylation of mT on tyrosine *in vivo* (Segawa and Ito, 1982). Furthermore, Hunter *et al.* (1981)

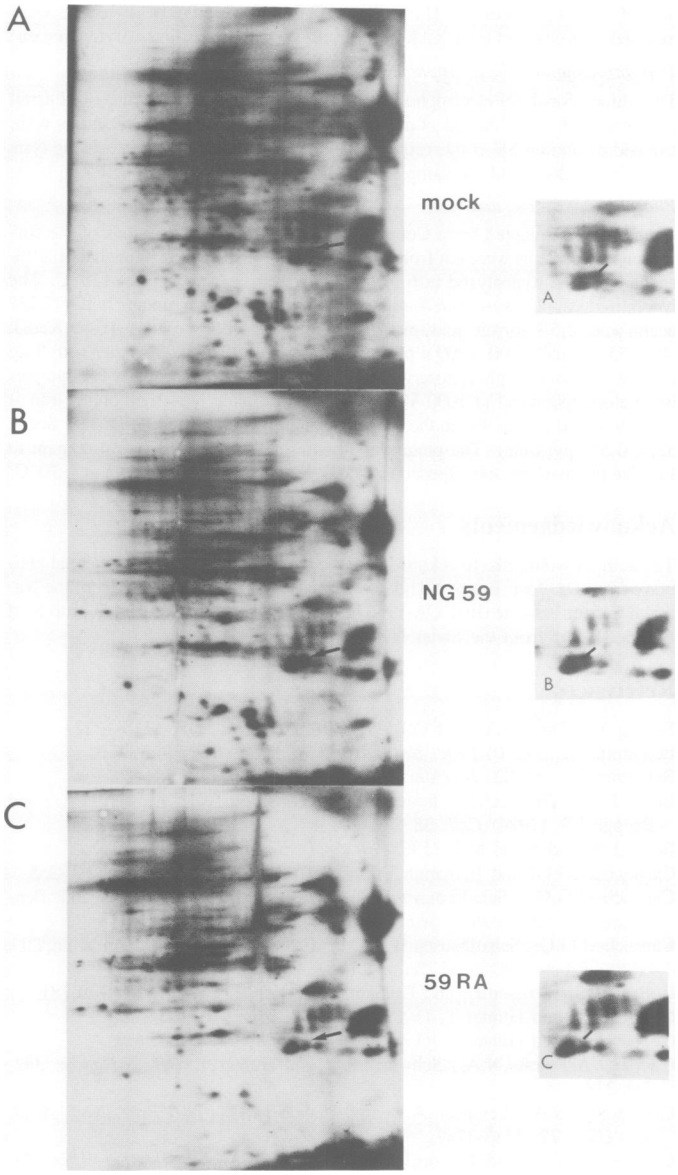


**Fig. 4.** Two-dimensional gels (pI 4–7; 10% acrylamide in the second dimension) of total [$\gamma$-$^{32}$P]ATP labeled membranes. **Left**: whole gels of membranes from mock-infected cells (**A**), *hr-t* mutant infected cells (**B**), and wild type infected cells (**C**). Arrows indicate the spot missing in wild-type virus-infected cell membranes. **Right**: sections of the whole gels showing the region around pI 5 and mol. wt. 40 kd is enlarged. Bar indicates the spot missing in wild-type infected cell membranes.

have shown that polyoma virus does not induce an increase in tyrosine phosphorylation of cellular proteins in infected cells. Substitution of phenylalanine for tyrosine at the major site of *in-vitro* phosphorylation in mT gives a mutant virus (1178T) with reduced transforming ability consistent with a biological role for this modification (Carmichael *et al.*, 1984). However, transfection of normal rat fibroblasts with an mT cDNA containing the same mutation shows normal induction of the transformed phenotype (Oostra *et al.*, 1983). It is clear that association of mT with membranes is essential for transformation (Carmichael *et al.*, 1982; Templeton and Eckhart, 1981), and with pp60$^{c\text{-}src}$ for phosphorylation *in vitro* (Courtneidge and Smith, 1983, 1984). Membrane attachment of mT is also important for efficient serine/threonine phosphorylation of 58 kd *in vivo* (Carmichael *et al.*, 1982).

We report here results of phosphorylation carried out on purified plasma membranes from polyoma-virus lytically infected cells. The mT and pp60$^{c\text{-}src}$ proteins together represent major alkali-stable phosphorylated species in these membranes as well as in membranes from polyoma virus-transformed cells. mT in isolated membranes is phosphorylated on tyrosine in the same manner as occurs in immunoprecipitates. Data from the same experiments suggest that pp60$^{c\text{-}src}$ undergoes autophosphorylation on tyrosine, possibly enhanced by the presence of a functional mT in the membranes. These results thus confirm and extend earlier work carried out on mT-pp60$^{c\text{-}src}$ complexes (Bolen *et al.*, 1984; Courtneidge and Smith, 1983, 1984; Yonemoto *et al.*, 1985), and further emphasize the potential importance of this interaction in transformation by polyoma virus.

Tyrosine phosphorylation in plasma membranes is very unstable and detection of labeled mT was only possible when vanadate was used to inhibit tyrosine-specific phosphatases during extraction and immunoprecipitation. Others have described membrane-bound phosphatase activity specific for phosphotyrosine (Gallis *et al.*, 1981; Leis and Kaplan, 1982). It is therefore conceivable that membrane-bound mT is phosphorylated *in vivo* on tyrosine, and that the tyrosine-linked phosphate is largely lost in the course of T-antigen isolation from intact cells.

The fact that in isolated plasma membranes mT is labeled poorly on serine and threonine can be explained in several ways: (i) serine/threonine phosphorylation may occur before or immediately after insertion of mT in the plasma membrane; (ii) the relevant serine/threonine protein kinases may be absent or inactive in isolated membranes; and (ii) the turnover of serine/threonine-linked phosphate on mT is so low that no rephosphorylation of sites exposed by the action of phosphatases during membrane preparation is possible. Further work is needed to distinguish among these possibilities.

Previous experiments have shown tyrosine phosphorylation of mT *in situ* in detergent-treated cell monolayers (Schaffhausen and Benjamin, 1981b; Schaffhausen *et al.*, 1982). While the bulk of metabolically-labeled mT is readily extracted by buffers containing non-ionic detergent, the kinase-active mT molecules are preferentially bound to cell frameworks (Schaffhausen *et al.*, 1982; Schaffhausen *et al.*, 1985; Segawa and Ito, 1982). We suggest that it is this subpopulation of mT molecules that preferentially interact with pp60$^{c\text{-}src}$. Sedimentation analysis has also shown that kinase-active mT molecules run as a complex more rapidly than the bulk of the metabolically-labeled mT molecules (Courtneidge and Smith, 1984; Schaffhausen and Benjamin, 1981b).

We have attempted to identify changes in phosphorylation of membrane-associated cellular proteins due to the presence of a functional mT protein. Several reports show that retroviruses induce significant changes in the phosphorylation of a variety of cellular proteins, mostly on tyrosine residues (Cooper and Hunter, 1981, 1982; Cooper *et al.*, 1983; Hunter *et al.*, 1981; Radke *et al.*, 1983). In purified plasma membranes of Rous sarcoma virus-transformed cells labeled with [$\gamma$-$^{32}$P]ATP, several proteins are more highly phosphorylated compared to uninfected cell membranes (Gallis *et al.*, 1981). Moreover, two *in-vivo* phosphate-labeled membrane proteins are rapidly dephosphorylated in the presence of an intact Rous sarcoma *src*-gene product in the membranes (Witt and Gordon, 1980). As shown in Figure 1A and 1B, no significant change in protein phosphorylation of labeled plasma membranes is observed using one-dimensional SDS gels. In two-dimensional gels, an acidic protein with a mol. wt. of ~40 kd appears in cell membranes of untransformed cells

but not in membranes of wild-type virus-infected cells. This protein may be missing entirely from membranes of wild-type virus-infected cells; alternatively it could be present in a fully and stably phosphorylated form unable to accept phosphate *in vitro*. The protein may also be present predominantly as a dephosphorylated species due to a phosphatase activated in wild-type virus-infected cells. Whether or not our observation reflects the actual situation *in vivo* must be further clarified. Our membrane system may provide the means to study the effect of mT on phosphorylation of membrane proteins in more detail.

## Materials and methods

*Cells and viruses*

NIH-3T3 cells were used. Virus stocks were grown on baby mouse kidney cells. NG59 and B2 were used as prototype *hr-t* mutants (Benjamin, 1970; Staneloni *et al.*, 1977). dl45 is a 'wild-type' strain which shows internally deleted middle and large T antigens (Bendig *et al.*, 1980). 1387T is a mutant virus that lacks the carboxy-terminal hydrophobic sequence of mT antigen (Carmichael *et al.*, 1982). NG59RA is a wild-type virus derived from NG59 by marker rescue (Feunteun *et al.*, 1976).

*Materials*

[$\gamma$-$^{32}$P]ATP was from ICN (7000 Ci/mM). Monoclonal antibody 327 reactive against pp60$^{src}$ was provided by Dr. Joan Brugge (Lipsich *et al.*, 1983). Anti-polyoma T ascites fluid was obtained as described previously (Schaffhausen *et al.*, 1978). Protein A Sepharose was from Pharmacia Fine Chemicals, Inc.

*Cell culture and preparation of plasma membranes*

Plasma membranes were made according to Thom *et al.* (1977). In brief, 10–30 10-cm tissue-culture Petri dishes were infected with virus at a multiplicity of 10 p.f.u. per cell. After 24 h they were washed with ice-cold phosphate-buffered saline (PBS) and isotonic borate solution (150 mM NaCl, 1 mM $Mg^{2+}$, 1 mM $Ca^{2+}$, 50 mM borate pH 7.2), scraped off the plate with a rubber policeman and centrifuged at 400 g for 5 min at 4°C. The cell pellet was resuspended in 1 ml of isotonic borate solution, and added slowly to 100 ml of hypotonic borate solution (20 mM borate, 0.2 mM EDTA, pH 10.2) with stirring in an Erlenmeyer. After 10 min, 8 ml of 0.5 M borate pH 10.2 was added. After stiring for 5 min, the suspension was filtered through two layers of nylon gauze ('Nytex' – 110 $\mu$m mesh, from Tetco). Nuclei were removed by centrifugation at 1500 g for 8 min, and the supernatant centrifuged in a type 35 rotor for 30 min at 15 000 r.p.m. The supernatant was discarded. The pellet was homogenized with a Dounce homogenizer in 13 ml of PBS, and layered onto a sucrose cushion (35% sucrose w/w in PBS, 25 ml) in an SW27 rotor tube. The samples were centrifuged for 45 min at 18 000 r.p.m. The membranes were collected from the interphase, diluted 1:1 in PBS and pelleted in a type 65 rotor for 30 min at 40 000 r.p.m. The pellets were dissolved in 20 mM Hepes pH 7.4, 10% glycerol at ~10 mg protein/ml, and stored in aliquots at −70°C. These preparations retained phosphorylating activity after storage for several months.

Crude membrane fractions were obtained by scraping infected cells into ice-cold hypotonic buffer (10 mM NaCl, 10 mM Hepes pH 7.4, 1 mM $Mg^{2+}$, 1 mM $Ca^{2+}$, 1 mM KCl). The cells were kept on ice for 10 min, disrupted in a Dounce homogenizer and the nuclei removed by spinning at 1500 r.p.m. for 8 min. The crude membranes were pelleted in a type 65 rotor at 40 000 r.p.m. for 20 min and dissolved in 20 mM Hepes pH 7.4, 10% glycerol.

*Phosphorylation of plasma membranes*

20 $\mu$g of membranes in a buffer containing 20 mM Hepes pH 7.4, 5 mM $MgCl_2$ and 10% glycerol were incubated at room temperature for 10 min in a volume of 20 $\mu$l. 5 $\mu$l of a solution containing 25 $\mu$M ATP and 20–300 $\mu$Ci [$\gamma$-$^{32}$P]-ATP were added for 4 min at room temperature. The reaction was stopped by adding 25 $\mu$l of two-fold concentrated SDS sample buffer for direct analysis of total proteins, or 10 volumes of NP40 lysis buffer (10 mM Tris pH 7.4, 1 mM $Mg^{2+}$, 1 mM $Ca^{2+}$, 137 mM NaCl, 1% w/w NP40, 100 $\mu$M $NaVO_3$, 10 mM NaF) for subsequent immunoprecipitation. For direct analysis, samples were dissolved in sample buffer, boiled, and loaded onto 10% polyacrylamide gels. Samples for immunoprecipitation were first dissolved in NP40 lysis buffer, incubated for 20 min on ice, cleared by centrifugation, and the supernatant incubated with anti-polyoma or anti-src serum. Immune complexes were collected on Staph A-Sepharose beads, washed, dissolved in sample buffer and run on 10% polyacrylamide gels.

*SDS acrylamide gel electrophoresis*

SDS gels were run as described by Laemmli (1970). Gels were fixed in methanol/acetic acid/water and either dried directly or treated for 1 h at 65°C in 1 M NaOH (Cooper and Hunter, 1981) and then dried. The dried gels were exposed to Kodak XOmat XAR5 film with or without an intensifying screen.

*Two-dimensional gel electrophoresis*

Two-dimensional gel electrophoresis was performed as described by O'Farrell (O'Farrell, 1975; O'Farrell *et al.*, 1977). The phosphorylation reactions were stopped by adding 5% $\beta$-mercaptoethanol and 0.5% SDS. Electrofocusing lysis buffer was added and the sample brought to 9 M urea and 5% NP40.

*Phosphoamino acid analysis*

The method is adapted from Cooper and Hunter (1981). The bands of the corresponding proteins were cut from the dried gels, washed in 45% methanol, 10% acetic acid and hydrolyzed in 6 N HCl under nitrogen for 2 h at 110°C. The lyophilized samples were then dissolved in the first-dimension running buffer (7.2% acetic acid, 2.5% formic acid, pH 1.9) and spotted onto a cellulose plate (Kodak # 13255). Usually 100–200 c.p.m. (Cerenkov) were spotted together with 5 $\mu$g each of a mixture of phosphoserine, threonine and tyrosine (Sigma). The samples were electrophoresed at 1000 V for 90 min. The plate was dried and re-run at 1000 V for 60 min at 90° to the first dimension in a buffer at pH 3.5 (5% acetic acid, 0.5% pyridine). The plate was dried, sprayed with ninhydrin reagent to localize the markers and exposed to Kodak XAR5 film with a screen at −70°C.

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