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Biochemistry

Tyrosine phosphorylation is a signal for the trafficking of pp85, an 85-kDa phosphorylated polypeptide associated with phosphatidylinositol kinase activity

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ABSTRACT **A family of 85/86-kDa (85K/86K) polypeptides closely linked to phosphatidylinositol kinase activity is found in polyoma middle-sized tumor antigen (MTAg)/pp60$^{c\text{-}src}$ complexes. MTAg and the 85-kDa phosphoprotein (pp85) could be reassociated in solution, or on blots, after denaturation with SDS. Results from such experiments focus attention on phosphorylation in controlling intracellular sorting and activation of pp85. Tyrosine phosphorylation seems important for recruitment of pp85 from cytosol to membrane. By blotting, pp85 is substantially cytosolic, whereas that recognized by anti-phosphotyrosine antibody is almost exclusively in membranes. Tyrosine phosphorylation also determines association of pp85 with MTAg. Manipulation of MTAg tyrosine phosphorylation, for example, by expressing MTAg using baculovirus vectors in the absence or presence of pp60$^{c\text{-}src}$, dramatically affects reassociation. Finally, tyrosine phosphorylation appears to be involved in release of pp85 from MTAg, since vanadate increased its rate of dissociation.**

The realization that oncogenes and growth factor receptors possess tyrosine kinase activity leads naturally to a search for relevant cellular substrates. Attention has been drawn to 85-kDa polypeptides because their phosphorylation upon activation of growth factor receptors and oncogenes parallels the activation of a phosphatidylinositol (PI) kinase. Both the polyoma virus middle-sized tumor antigen (MTAg)/c-src complex (1) and the platelet-derived growth factor receptor (2–4) have PI kinase activity associated with them. There is an excellent correlation between the ability of MTAg to transform and the presence of PI kinase activity (5). PI kinase activity and the 85-kDa phosphoprotein (pp85) polypeptides can be partially copurified (3); however, proof that the polypeptide has enzymatic activity is lacking.

The pp85 polypeptides actually represent a complex set of at least 10 species (6). There are two families, pp85/pp86, separated by about 1 kDa; the ratio of the two families depends on the cell type. Each set can be resolved into at least five forms by isoelectric focusing (IEF). These largely represent tyrosine- and serine-phosphorylated forms (3, 6). The rapid association of these peptides with MTAg/pp60$^{c\text{-}src}$ is complete within 10 min of their synthesis and is reversible. The association of 85-kDa species with MTAg/pp60$^{c\text{-}src}$ or the platelet-derived growth factor receptor coincides with its increased phosphorylation on tyrosine. The natural hypothesis is that tyrosine phosphorylation of pp85 by an activated tyrosine kinase leads to an increased PI kinase activity. Consistent with this hypothesis, a decrease in PI kinase activity has been seen upon phosphatase treatment (7).

The work presented here further characterizes the pp85 polypeptides and their interaction with polyoma MTAg. We present a method for reassociating MTAg with pp85 *in vitro*. These methods, in combination with immunoprecipitation results, present a picture emphasizing the importance of phosphorylation in the trafficking of the pp85 polypeptides.

MATERIALS AND METHODS

Cells and Viruses. NIH 3T3, MTAg-transformed 3T3 (MT-3T3), Rat-1, and Rat-1 cells transformed by either wild-type polyoma or Py1178T (8) MTAg were all carried in Dulbecco's modified Eagle's medium containing 10% (vol/vol) calf serum. MTAg- and pp60$^{c\text{-}src}$-expressing baculoviruses, as described (9), were propagated on Sf9 cells in Grace's medium containing 10% (vol/vol) fetal bovine serum. Infected cells were used 40 hr after infection as a source of MTAg. Rous sarcoma virus-transformed turkey embryo fibroblasts (B.C. and B.S.S., unpublished results) were carried in 5% fetal calf serum.

Labeling and Immunoprecipitations. The procedures for radiolabeling of cells, antigen extraction, and immunoprecipitation have been published in detail (10, 11). [^{35}S]Methionine labeling was in Hanks' salts (Flow Laboratories) containing [^{35}S]methionine (at 100–500 μCi/ml; 1 Ci = 37 GBq; New England Nuclear) for 90 min. $^{32}PO_4$ labeling was in phosphate-free medium containing $^{32}PO_4$ at 200–1000 μCi/ml. Antigens were routinely extracted in Nonidet P-40 (NP-40)-containing buffer and incubated with antiserum and protein A-Sepharose (Pharmacia) for 1 hr. Rabbit anti-small tumor antiserum (anti-T) (12) was generally used. Anti-phosphotyrosine monoclonal was derived by Brian Druker (B. Druker, D. Morrison, and M. White, personal communication). For some experiments, washed immunoprecipitates were treated with an SDS dissociation buffer at 100°C for 2 min (11). For others, tumor antigens were subjected to a second round of immunoprecipitation using a method similar to that devised for nuclear lamins (13) and modified for use with topoisomerase II (14). Immunoprecipitates were boiled for 2 min in a solubilization buffer containing 0.4% SDS, 50 mM triethanolamine chloride (pH 7.4), 100 mM NaCl, 2 mM EDTA (pH 7.4), and 2 mM 2-mercaptoethanol. After boiling, iodoacetamide was added to 10 mM. Finally 0.25 vol of 10% (vol/vol) Triton X-100 was added. These extracts were again immunoprecipitated.

For protein phosphorylation, immunoprecipitates washed in 10 mM Tris·HCl, pH 7.5/5 mM $MnCl_2$ were incubated with [γ-^{32}P]ATP for 20 min at room temperature (15). PI kinase assays were as described (1). Immunoprecipitates were in-

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Abbreviations: PI, phosphatidylinositol; MTAg, middle-sized tumor antigen; pp, phosphoprotein; NP-40, Nonidet P-40; IEF, isoelectric focusing.
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cubated in TNE buffer (20 mM Tris·HCl, pH 7.4/100 mM NaCl/1 mM EDTA) with 10 μg of sonicated phospholipid [PI/phosphatidylserine, 1:1 (wt/wt)] and [γ-^{32}P]ATP for 15 min at room temperature. Lipids were extracted with $CHCl_3$/MeOH, 1:1 (vol/vol), and resolved on a TLC plate (Silica Gel 60 without indicator treated with 1% potassium oxalate) developed in $CHCl_3/MeOH/H_2O/NH_4OH$, 45:35:8.5:1.5 (vol/vol).

***In Vitro* Reassociation.** Washed immunoprecipitates labeled with [γ-^{32}P]ATP *in vitro* were mixed with washed unlabeled immunoprecipitates from MT-3T3 cells phosphorylated *in vitro* using 5 mM unlabeled ATP. The combined pellets were boiled in 200 μl of solubilization buffer containing 2 mM 2-mercaptoethanol for 2 min. Triton X-100 and iodoacetamide were added as above and MTAg and associated proteins were then reimmunoprecipitated.

MTAg Blotting Procedure. Proteins were transferred to nitrocellulose by electroblotting (16). The nitrocellulose was washed for two 10-min periods in buffer (0.4% SDS/50 mM triethanolamine, pH 7.4/100 mM NaCl/2 mM EDTA) to which 0.25 vol of 10% Triton X-100 had been added. The blot was blocked in the same buffer containing 1% gelatin for 30–60 min. For probe, anti-T immunoprecipitate [9 μl of anti-T and 300 μl of 50% (wt/vol) protein A-Sepharose] from a single 100-mm dish of Sf9 cells expressing MTAg and pp60$^{c\text{-}src}$ was labeled with [γ-^{32}P]ATP. Labeled MTAg was boiled in SDS and reimmunoprecipitated. The second immunoprecipitate was boiled three times in 300 μl of solubilization buffer containing 2 mM 2-mercaptoethanol to extract all of the labeled MTAg. Then 0.25 vol of 10% Triton X-100 and 0.5 M iodoacetamide to a final concentration of 10 mM were added to the final extract. The labeled MTAg was diluted to 10–15 ml with solubilization buffer containing 0.25 volume of Triton X-100 lacking 2-mercaptoethanol and used to probe the nitrocellulose in sealed plastic bags for 12 hr at 4°C. The nitrocellulose was washed for three to five 20-min periods in solubilization buffer containing 0.25 vol of 10% Triton X-100 but without 2-mercaptoethanol. The nitrocellulose blot was air-dried and exposed on XAR5 film.

Electrophoresis. Samples were analyzed on discontinuous buffer SDS gels (17). IEF samples were prepared by addition of Garrels' sample buffer {9.5 M urea/2% (vol/vol) ampholytes (LKB) [pH 3-10/pH 5-7/pH 6-8, 1:2:2 (vol/vol)]/100 mM dithiothreitol/4% NP-40} (18). After incubation on ice for 30 min, the samples were loaded onto IEF cylinders. First-dimension 15-cm IEF gels were composed of 4% polyacrylamide [*N,N*′-methylenebisacrylamide/acrylamide, 1.62:28.3% (wt/vol)], 8.0 M urea, 4% NP-40, and 2% ampholytes as above. Samples were electrophoresed at 400–500 V until 9000 V·hr and then turned up to 800 V for 1 hr. Second-dimension PAGE was on 5% or 7.5% polyacrylamide discontinuous SDS buffer slab gels.

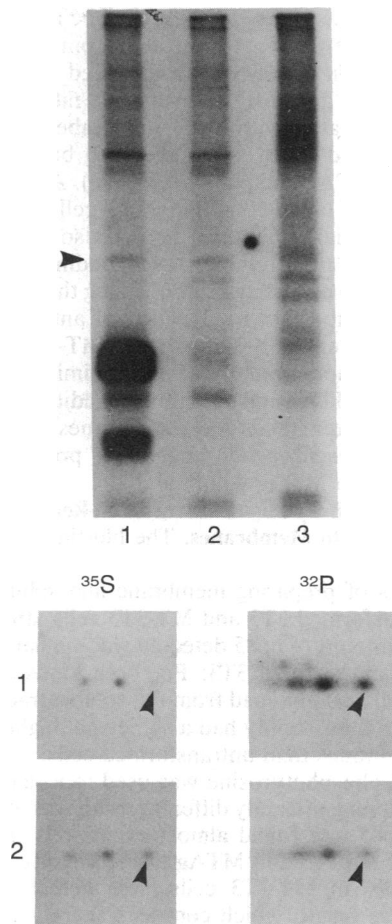

FIG. 1. Reassociation of MTAg with pp85 in solution. (*Upper*) Unlabeled anti-T immunoprecipitates from MTAg-transformed 3T3 cells were mixed with *in vitro* [γ-^{32}P]ATP-labeled anti-T immunoprecipitates from MTAg-transformed 3T3 cells (lane 1), anti-phosphotyrosine immunoprecipitates from 3T3 cells (lane 2), or anti-src immunoprecipitates from pp60$^{v\text{-}src}$-transformed turkey embryo fibroblasts (lane 3) and boiled in SDS. The denatured mixture was reimmunoprecipitated with anti-T. The proteins were resolved by discontinuous SDS/PAGE on a 7.5% gel. The arrowhead indicates pp85. The dark band at the bottom of lane 1 represents labeled MTAg. (*Lower*) IEF of reassociated pp85. Immunoprecipitated proteins from MTAg-transformed 3T3 cells either labeled *in vivo* with [^{35}S]methionine or *in vitro* with [γ-^{32}P]ATP were divided. One portion (gels 1) of each was immediately subjected to two-dimensional IEF analysis. The other (gels 2) was denatured, incubated with unlabeled MTAg, and then immunoprecipitated again prior to gel analysis. The pp85 region is shown; the most basic species is indicated by an arrowhead.

RESULTS

Reassociation of MTAg with pp85 Can Be Carried Out in Solution and on Blots. Fig. 1 shows reassociation of MTAg with pp85 in solution. Anti-T immunoprecipitates from MTAg-transformed 3T3 cells, anti-phosphotyrosine immunoprecipitates from nontransformed 3T3s, and anti-v-src immunoprecipitates from Rous sarcoma virus-transformed primary turkey embryo fibroblasts were labeled *in vitro* with [γ-^{32}P]ATP. Previous work has shown that pp85 is phosphorylated in each of these immunoprecipitates (3, 6). After mixing each of these with unlabeled MTAg, denaturation was carried out by boiling in SDS/2-mercaptoethanol. The SDS was titrated out with excess of Triton X-100 (19). The samples were then reimmunoprecipitated with fresh anti-T serum. Not only MTAg but also reassociated labeled pp85 were reimmunoprecipitated as well (lane 1). The identity of pp85 has been confirmed by partial peptide mapping (data not shown) and by IEF (Fig. 1 *Lower*). IEF showed that both modified and unmodified pp85 reassociated but that there is some preference for the more-basic, less-phosphorylated forms. Reassociation was primarily dependent upon MTAg. If reimmunoprecipitation was carried out with anti-src (20), then much less pp85 was obtained. pp85 labeled in anti-phosphotyrosine immunoprecipitates from nontransformed 3T3 cells also associated with MTAg, showing that no modification of pp85 occurring in the transformed cell was required for reassociation. Avian pp85/86, which shows a more even distribution of pp85/86 forms (6), also reassociated with MTAg, even when originally derived from pp60$^{v\text{-}src}$ immunoprecipitates. Although bands other than pp85 were observed after reassociation, these were nonspecific, appearing regardless of whether MTAg or anti-T serum was added prior to the second immunoprecipitation.

MTAg/pp85 reassociation could also be accomplished on a blot. Increasing amounts of protein from nontransformed 3T3 cells or *Escherichia coli* were loaded on a 7.5% polyacrylamide/SDS gel. After transfer to nitrocellulose, the blots were incubated with [γ-^{32}P]ATP-labeled MTAg from Sf9 cells that had been coinfected with baculoviruses expressing both MTAg and pp60$^{c\text{-}src}$ (Fig. 2). A band at 85 kDa was observed in lanes containing 3T3 cell protein but not those with bacterial protein. pp85 could also be detected with labeled MTAg at the pI value corresponding to the unmodified form of pp85 on IEF gels, confirming the blotted species was pp85 (data not shown). Blotting of anti-T immunoprecipitates from an entire 100-mm dish of MT-3T3 cells did not detect pp85, demonstrating that only a limited fraction was associated with MTAg in those cells. In addition to prominent pp85 bands, fainter bands have sometimes been observed; these may represent pp85 breakdown products or other proteins to which MTAg will bind.

Tyrosine Phosphorylation Is Involved in Recruitment of pp85 from the Cytosol to Membranes. The blotting procedure was used to test the subcellular distribution of pp85. Fig. 3 *Upper* shows results of preparing membrane and soluble fractions from nontransformed 3T3 and MT-3T3 cells after hypotonic shock. The amount of pp85 detected was similar for untransformed and transformed 3T3. For both kinds of cells, the greatest signal was obtained from the soluble fraction. MTAg transformants consistently had a somewhat higher portion of pp85 in membranes than untransformed cells.

When anti-phosphotyrosine was used to isolate pp85 after fractionation, a significantly different result was obtained (Fig. 3 *Lower*). pp85 was found almost exclusively in membrane fractions in both 3T3 and MTAg transformants. Little material, especially in MT-3T3 cells, was detected in soluble fractions. This result, which contrasts sharply with that obtained by blotting, suggests that tyrosine phosphorylation is a basis for recruitment of pp85 to membranes. IEF patterns showed that both phosphorylated and unphosphorylated pp85 were found in membrane fractions, implying an interaction of unmodified pp85 with tyrosine-phosphorylated proteins.

Phosphorylation of MTAg Affects Association with pp85. Several experiments suggested that phosphorylation of MTAg, particularly on tyrosine-315, is important for the association with pp85. When MTAg alone was expressed using the baculovirus expression system, little if any tyrosine phosphorylation on residue 315 was observed; when MTAg and pp60$^{c\text{-}src}$ are coexpressed in insect cells, then MTAg is phosphorylated on residue 315 (data not shown). Whereas comparable amounts of MTAg could be immunoprecipitated from either infection (Fig. 4*A*, lanes 1 and 2), only the tyrosine-phosphorylated MTAg (lane 2) associated efficiently with pp85. Interestingly, pp60$^{c\text{-}src}$ did not work well in

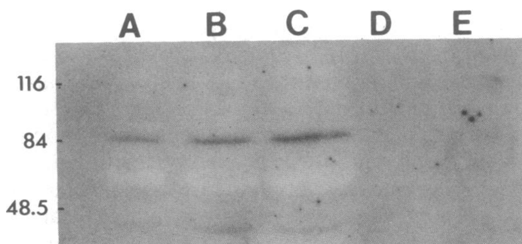

FIG. 2. Renaturation on blots. NP-40 extracts of 10% (lane A), 30% (lane B), and 50% (lane C) of a 100-mm dish of MTAg-transformed 3T3 cells were electrophoresed on a 7.5% polyacrylamide/SDS gel. Amounts of bacterial protein (lanes D and E) equivalent to those of lanes B and C, respectively, were included as controls. Proteins were transferred to nitrocellulose and probed with *in vitro* ^{32}P-labeled MTAg. The positions of the molecular mass standards are shown in kDa.

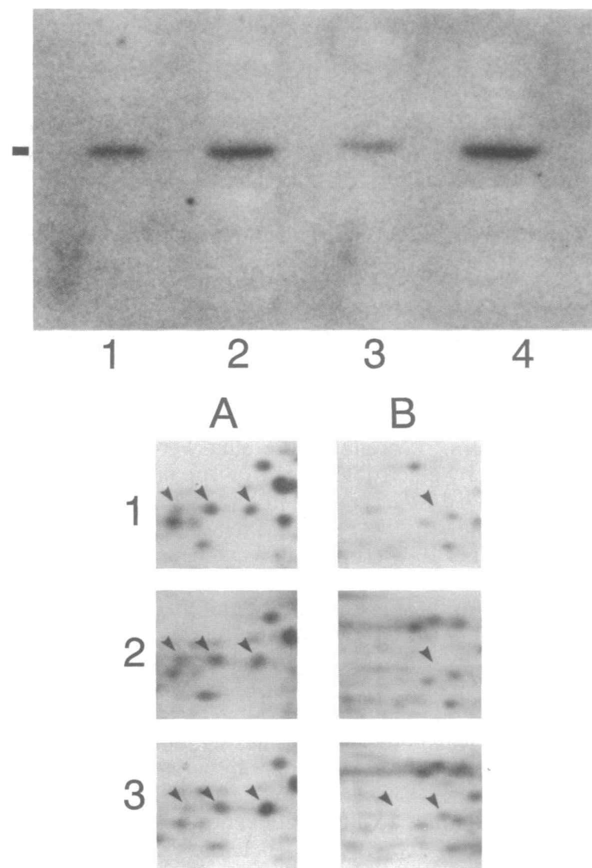

FIG. 3. Subcellular localization of pp85. (*Upper*) Nontransformed 3T3 (lanes 3 and 4) and MTAg-transformed 3T3 (lanes 1 and 2) cells were separated into membrane (lanes 1 and 3) and cytosolic (lanes 2 and 4) fractions by differential centrifugation after hypotonic shock (11). Protein extracted by NP-40 was electrophoresed, transferred to nitrocellulose, and probed with ^{32}P-labeled MTAg. The pp85 shown is indicated by the tick mark. (*Lower*) 3T3 (gels 3) and MT-3T3 (gels 1 and 2) cells labeled with [^{35}S]methionine for 2 hr were fractionated as above. The membrane and cytosolic fractions were extracted with 1% NP-40 and immunoprecipitated with anti-T (gels 1) and anti-phosphotyrosine (gels 2 and 3). Immunoprecipitated proteins were then resolved by IEF using a 5% polyacrylamide second-dimension SDS gel. The most basic unphosphorylated form of pp85 is to the right. Its position, whether a spot is present (gels A) or not (gels 1B), is indicated in each panel by an arrowhead; phosphorylated forms are indicated by the presence of additional arrowheads.

reassociation (lane 3), even though it is phosphorylated on tyrosine-416 and activated (9). Treatment of MTAg with potato acid phosphatase prior to reassociation resulted in a decreased pp85 reassociation (Fig. 4*B*). Transformation defective Py1178T MTAg lacks tyrosine-315 (21). Little if any pp85 was found to be reassociated with this mutant MTAg (Fig. 4*C*), even though Western blot analysis showed that wild-type and Py1178T-expressing cells had similar amounts of protein (data not shown).

Tyrosine Phosphorylation Affects Dissociation of the MTAg–pp85 Complex. The association of pp85 with MTAg/pp60$^{c\text{-}src}$ is rapid and reversible as shown by pulse–chase experiments (6). Vanadate, an inhibitor of tyrosine phosphatases (22), had little effect on total pp85 associated with MTAg when added during a pulse period (Fig. 5). However, the presence of vanadate during a chase period *in vivo* dramatically accelerated its release from MTAg.

Py1178T Suggests Another Possible Role for Tyrosine Phosphorylation *in Vivo*. Detailed analysis of Py1178T provides an interesting confirmation of roles of tyrosine phosphorylation just described. Py1178T MTAg did not reassociate with pp85

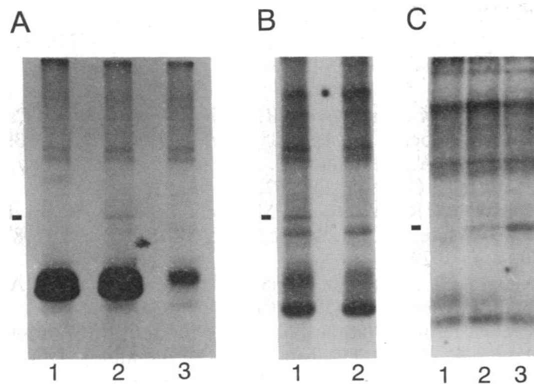

FIG. 4. Phosphorylation of MTAg by pp60^{c-src} may play a critical role in the formation of MTAg/85-kDa complex. (*A*) Reassociation depends on tyrosine phosphorylation of MTAg. Immunoprecipitates were made from Sf9 cells labeled *in vivo* with [γ-^{32}P]ATP expressing MTAg (lane 1), MTAg and pp60^{c-src} (lane 2), or pp60^{c-src} alone (lane 3) using baculovirus vectors. One sample was used to check MTAg phosphorylation (data not shown). A second was mixed with [γ-^{32}P]ATP-labeled anti-phosphotyrosine immunoprecipitates from 3T3 cells. The mixture was boiled in SDS and MTAg was reimmunoprecipitated with anti-T serum (lanes 1 and 2) or anti-src (lane 3). The resulting immunoprecipitate was washed, boiled in SDS dissociation buffer, and analyzed by SDS/PAGE. (*B*) Phosphatase treatment of MTAg results in a decreased level of reassociated 85-kDa protein. Baculovirus-infected Sf9 cells expressing MTAg and pp60^{c-src} were extracted and the MTAg was immunoprecipitated with anti-T antibody. The immunoprecipitate was divided and one part was treated with potato acid phosphatase (lane 2) and the other was not (lane 1). The resulting washed immunoprecipitates were mixed with *in vitro* [γ-^{32}P]ATP-labeled anti-phosphotyrosine immunoprecipitates from 3T3 cells and boiled in SDS. MTAg was then reimmunoprecipitated and the proteins were resolved on SDS/PAGE. (*C*) MTAg of Py1178T does not reassociate efficiently with pp85. Anti-phosphotyrosine immunoprecipitates from 3T3 cells were labeled *in vitro* with [γ-^{32}P]ATP. This material was reassociated with MTAg immunoprecipitates from Py1178T-carrying Rat-1 (lane 1), wild-type MTAg-carrying Rat-1 (lane 2), or MTAg-transformed 3T3 (lane 3) cells. (*A–C*) The position of pp85 is shown by the tick mark.

(Fig. 4*C*) and, when cells expressing Py1178T were pulse-labeled with [^{35}S]methionine, labeled pp85 failed to associate with Py1178T MTAg. Nonetheless, in apparent conflict with these observations, pp85 could be detected in association with Py1178T MTAg by labeling immunoprecipitates *in vitro* with [γ-^{32}P]ATP (Fig. 6). V8 peptide mapping has confirmed its identity (data not shown). A satisfactory explanation for the apparent contradiction emerges from the pp85 IEF pattern: pp85 associated with Py1178T MTAg is not as highly phosphorylated as pp85 from wild type. Although slower association (lack of pulse labeling) would be expected from mutation of tyrosine-315 in Py1178T, slower dissociation would also be expected to result from the lack of phosphorylation, permitting the accumulation of some pp85 with MTAg.

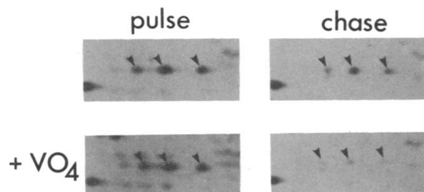

FIG. 5. *In vivo* treatment of cells with vanadate increases the dissociation of pp85 from the MTAg/pp60^{c-src} complex. (*Lower left*) MTAg-transformed 3T3 cells were incubated in 100 μM vanadate for 60 min and then pulse-labeled with [^{35}S]methionine also containing 100 μM vanadate for 20 min. (*Lower right*) The off-rate was analyzed by pulse-labeling the cells with [^{35}S]methionine containing 100 μM vanadate for 45 min and then chasing in the presence of vanadate for 20 min. (*Upper*) Control samples were treated in the same way except that vanadate was not used. pp85 associated with the MTAg/pp60^{c-src} complex was extracted and immunoprecipitated with anti-T serum. Labeled proteins were resolved by two-dimensional IEF gel electrophoresis.

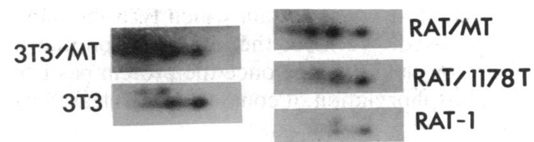

FIG. 6. IEF of the 85-kDa protein from wild-type or mutant MTAg-transformed mouse and rat cells. Anti-phosphotyrosine immunoprecipitates from 3T3 (*Left*) and Rat-1 (*Right*) cell lines and anti-T immunoprecipitates from MT-3T3 or Rat-1 cells expressing wild-type (MT) or Py1178T (1178T) MTAgs were labeled *in vitro* with [γ-^{32}P]ATP. pp85 was resolved by two-dimensional IEF gel electrophoresis. The most basic forms of the pp85 shown are to the right of the gel.

The lack of phosphorylation of pp85 associated with Py1178T MTAg suggests another role for tyrosine phosphorylation. It is possible that tyrosine phosphorylation of pp85 renders it a substrate for serine phosphorylation. We have shown (3) that most phosphate on pp85 is serine phosphate. The pp85 associated with the MTAg of Py1178T must be defective in serine phosphorylation as well. Alternatively, MTAg Py1178T, which lacks tyrosine phosphorylation, could be defective in activating cellular serine kinases responsible for phosphorylation of pp85.

DISCUSSION

pp85 can be phosphorylated by both platelet-derived growth factor receptor and the MTAg/pp60^{c-src} complex (3). MTAg and pp85 can be reassociated *in vitro*. In cells pp60^{c-src} is present in the complex as well, since pp85 can be immunoprecipitated by anti-src antibodies. Also, MTAg monoclonal antibody pAB815 (23) that recognizes MTAg/pp60^{c-src} complex poorly is also inefficient at recognizing MTAg associated with pp85. The role of pp60^{c-src}, if any, in reassociations *in vitro* cannot be definitively assessed. MTAg does not efficiently reassociate with pp60^{c-src}; pp60^{c-src}, for example, is not detected in blots with MTAg. The MTAg used in blotting should not contain MTAg/pp60^{c-src} complexes. However, after reassociation, a limited amount of MTAg/pp85 can be immunoprecipitated with anti-src antibody, indicating that some tripartite complex does form. The relative affinity of such MTAg/pp60^{c-src} complexes for pp85 could be higher than that of MTAg alone. Interestingly, neither activated pp60^{c-src} nor pp60^{v-src} functions in reassociation. Since pp85 is associated with pp60^{v-src} (6) *in vivo*, there may be an equivalent to MTAg acting to "broker" interaction between pp85 and pp60^{v-src}. However, renaturation may also be simply inadequate to permit reassociation.

Tyrosine phosphorylation is important for the association of pp85 with MTAg. Several lines of evidence point to the importance of MTAg tyrosine-315 in promoting association. MTAg lacking tyrosine phosphorylation (either because no pp60^{c-src} is available, as in the baculovirus-infected Sf9 cells, or because phosphate has been removed by phosphatase) functions poorly in the reassociation. Py1178T, which has a phenylalanine replacing tyrosine-315, is known to be defective in associated PI kinase activity when isolated from 3T3 cells (5). Py1178T MTAg did not function in the reassociation reaction. A similar conclusion has recently been reached for PI kinase activity using antibodies to the sequence around residue 315 (26). Finally, the enhanced release of pp85 from MTAg in the presence of vanadate suggests that tyrosine phosphorylation has a role in dissolution of complex. Since all three proteins (MTAg, pp60^{c-src}, and pp85) are tyrosine

phosphorylated, it is not certain which tyrosine phosphorylation is involved. We favor the interpretation that it is the pp85 phosphorylation, since once the protein has been activated by phosphorylation, it could then be released to act on substrate.

Tyrosine phosphorylation also appears to be a key signal for the cellular trafficking of pp85. In subcellular fractionation, pp85 was found largely in soluble fractions when assayed by protein blotting but was found in membrane fractions when immunoprecipitations were carried out with anti-phosphotyrosine. IEF suggests that the tyrosine phosphorylation need not necessarily be on pp85 itself. Unphosphorylated pp85 is also found in membranes. Association of pp85 with other tyrosine-phosphorylated proteins seems therefore likely, although pp85 could instead exist as dimers or other self-associated forms or there could be other subunits of the PI kinase. Certainly, MTAg would be among those in MTAg transformants and other membrane proteins such as growth factor receptors would be candidates as well. A specific conclusion has been reached about the importance of tyrosine phosphorylation for substrate binding to the platelet-derived growth factor receptor (24).

What is the significance of the observation that pp85 associated with Py1178T is not hyperphosphorylated on either serine or tyrosine residues? As expected, little PI kinase activity is found in such complexes. The observation also raises two interesting possibilities. One is that tyrosine phosphorylation by MTAg/pp60$^{c\text{-}src}$ activates pp85 as a substrate for subsequent serine phosphorylation. Alternatively, Py1178T MTAg may fail to activate a specific serine/threonine kinase (or to inactivate a phosphatase) ordinarily acted upon by wild type. The two possibilities are clearly not mutually exclusive. Simple membrane association of pp85, which might have been imagined to signal serine phosphorylation, must not be sufficient, since Py1178T MTAg and its complexed pp85 are associated with membranes.

Besides their value in deciphering the behavior of pp85, the methods used here may themselves be important for others trying to detect protein–protein interactions. The detection is akin to using nucleic acid sequences as a probe for DNA-binding proteins. Labeled calmodulin has been used in a similar sort of assay (25). Here we have used SDS-denatured, and then partially renatured, protein as a probe. We were able to detect pp85 from as little as 50 μg of total cell protein.

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