

Modulation of p36 Phosphorylation in Human Cells: Studies Using Anti-p36 Monoclonal Antibodies

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We have characterized two monoclonal antibodies which recognize human p36. These have been used to examine the sites and extent of serine and tyrosine phosphorylation of p36 in human cells treated with epidermal growth factor and platelet-derived growth factor and in human cells transformed with viruses whose oncogenes encode protein-tyrosine kinases.

The fact that some growth factor receptor genes and viral oncogenes both encode protein-tyrosine kinases suggests that protein modification as a result of tyrosine phosphorylation is important in the transduction of growth regulatory signals (reviewed in reference 31). One of the best-studied physiological substrates of protein-tyrosine kinases is the 36-kilodalton protein p36 (reviewed in reference 9). p36 was initially identified as a phosphoprotein (pp36), which was present in chicken fibroblasts transformed by Rous sarcoma virus (RSV) but not in the nontransformed parental cells (35). Subsequently it has been shown that the transformation of fibroblasts with a wide variety of viruses which encode functional protein-tyrosine kinases results in the phosphorylation of p36 on tyrosine residues (6, 7, 14, 15, 34). Here we report the generation and characterization of monoclonal antibodies (MAbs) against p36 from human fibroblasts and their use to reexamine the phosphorylation of p36 in human cells.

Characterization of MAbs against human p36. Hybridomas were produced by the fusion of Sp2/0 myeloma cells with spleens from mice immunized with either whole live human AG1523 diploid fibroblasts or crude membrane preparations (C. M. Isacke, C. A. Sauvage, R. Hyman, J. Lesley, R. Schulte, and I. S. Trowbridge, *Immunogenetics*, in press). Supernatants were screened by immunoprecipitation as previously described (Isacke et al., in press), and two hybridomas producing antibodies against a 36-kilodalton protein were subcloned to yield two stable lines, D1/274.5 and B1/107.36, both of which secreted immunoglobulins of the IgG_{2A} subclass. To confirm that the MAbs were directed against human p36, AG1523 cells were labeled with [³⁵S]methionine and lysed, and immunoprecipitates prepared with MAb D1/274, MAb B1/107, a polyclonal anti-chicken p36 serum known to be broadly species cross-reactive (8), or an anti-chicken p36 MAb which does not cross-react with human p36 (25) were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Fig. 1A). Two-dimensional tryptic peptide mapping of ³⁵S-labeled proteins showed that MAbs D1/274 and B1/107 recognized the same protein and that this protein was identical to p36 precipitated by the polyclonal antiserum (data not shown).

It has been reported that cells contain, in addition to p36, a related protein-tyrosine kinase substrate termed p35 (18, 21, 41; J. R. Glenney, Jr., *Proc. Natl. Acad. Sci. USA*, in press). Due to the confusion in nomenclature of the two

related proteins and the fact that p35 resolves in SDS-polyacrylamide gels as a larger protein than does p36, it has been proposed that they be called calpactin-I (p36) and calpactin-II (p35) (Glenney, in press). An immunoprecipitate from [³⁵S]methionine-labeled A431 cells (a sample of which is shown in Fig. 3A) was mixed with an extract from unlabeled cells enriched for calpactins and resolved by two-dimensional nonequilibrium pH gradient electrophoresis. MAb D1/274 precipitated a protein that comigrated with the faster-migrating, more acidic protein of the pair, which has been shown to correspond to p36 (Fig. 1B) (Glenney, in press). There was no [³⁵S]methionine-labeled protein comigrating with the Coomassie-stained spot identified as p35. A 10-fold-longer exposure of this autoradiogram still failed to reveal any cross-reaction of MAb D1/274 with p35, indicating the high degree of specificity of this MAb for p36.

Neither MAb cross-reacted with chicken p36; MAb B1/107 reacted weakly with rat and bovine p36 but not murine p36, whereas MAb D1/274 did not recognize p36 from any of these species (data not shown). Neither MAb detected denatured p36 as assayed by immunoblotting, but both displayed the same immunofluorescence pattern as anti-p36 serum in fixed, permeabilized AG1523 cells (data not shown).

Phosphorylation of p36 in response to growth factor treatment. When growing AG1523 cells were labeled for 18 h to equilibrium with ³²P_i, p36 was basally phosphorylated exclusively on serine residues (Fig. 2A and B). These cells express high-affinity platelet-derived growth factor (PDGF) receptors on their cell surface (2, 5, 28) and respond to PDGF mitogenically, as measured by a stimulation of [³H]thymidine incorporation (2; C. M. Isacke, unpublished results). A 10-min treatment of the cells before lysis with PDGF sufficient to saturate the PDGF receptors resulted in a twofold increase in the level of phosphorylation of p36 (Fig. 2A). Phosphoamino acid analysis showed that this increase was due partly to the appearance of phosphotyrosine (Fig. 2B). AG1523 cells also possess high-affinity receptors for epidermal growth factor (EGF). These receptors become phosphorylated upon treatment of membrane preparations with EGF (4, 6, 13; Isacke, unpublished results), and they respond mitogenically (26, 29). However, treatment with EGF did not result in a detectable increase in the phosphorylation of p36 at serine or tyrosine residues (Fig. 2A and B).

³²P-labeled p36 was excised from gels and digested with trypsin, and the phosphopeptides were resolved in two

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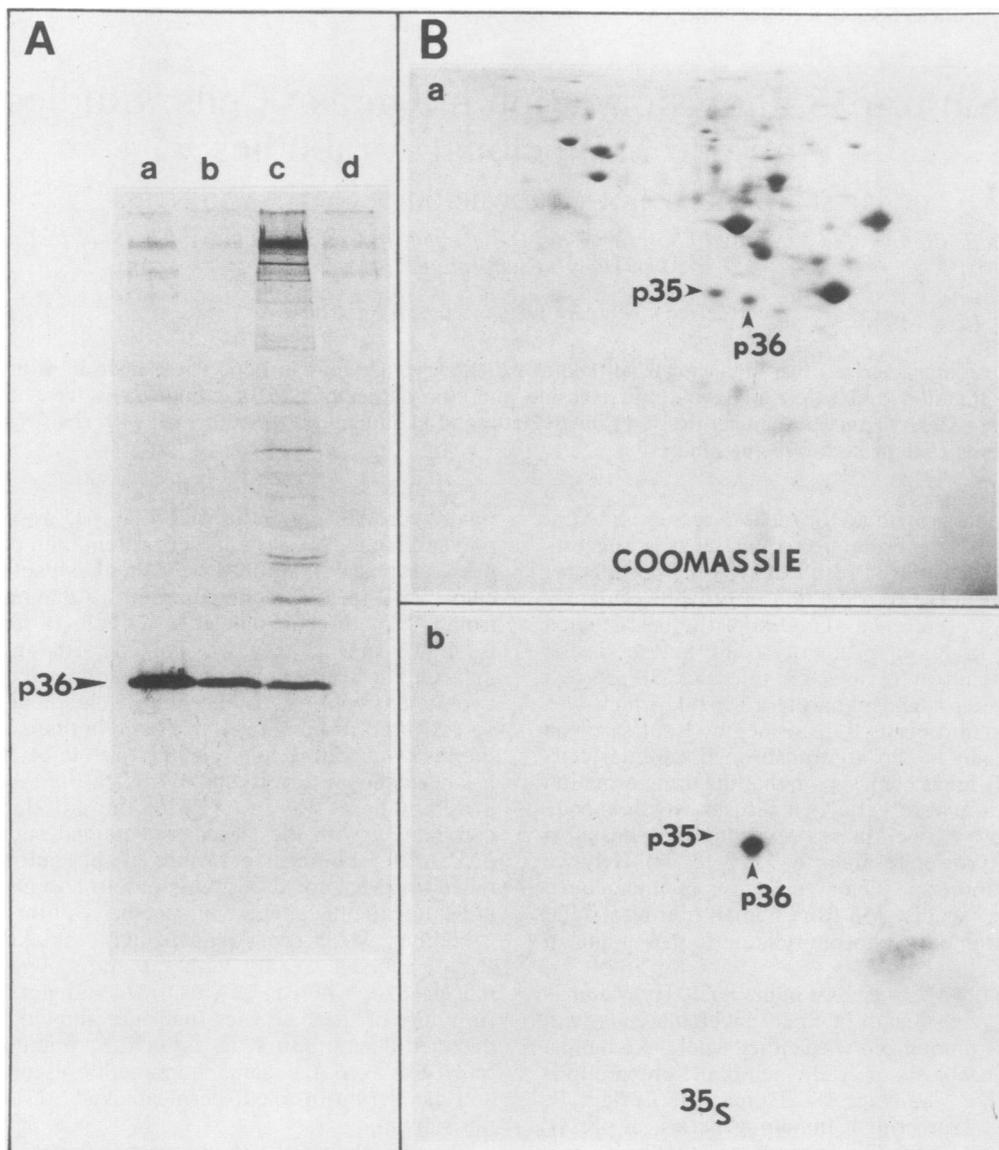


FIG. 1. Immunoprecipitation of p36 from human cells. (A) A 60-mm dish of AG1523 diploid human fibroblasts (NIA Aging Cell Repository, Institute of Medical Research, Camden, N.J.) plated 1 day previously was incubated for 18 h in 2 ml of methionine-free Dulbecco minimal essential medium containing 4% dialyzed fetal bovine serum and 300 μ Ci of [35 S]methionine ($>1,000$ Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per ml. Cells were lysed in 0.5 ml of RIPA buffer (44) containing 2 mM EDTA, 100 μ M Na_3VO_4 , and 50 mM NaF. The lysates were precleared for 30 min with 1% (vol/vol) fixed *Staphylococcus aureus* bacteria (Pansorbin; Calbiochem-Behring, La Jolla, Calif.), divided into four parts, and immunoprecipitated with the following: lane a, MAb D1/274; lane b, MAb B1/107; lane c, an anti-chicken p36 polyclonal antiserum (8); lane d, an anti-chicken p36 MAb (MAb 5E1) (25) as described by Sefton et al. (44). Immunoprecipitates were analyzed on a 15% SDS-polyacrylamide gel. Exposure time to Kodak XAR X-ray film was 16 h. (B) Resolution of [35 S]methionine-labeled p36 on a nonequilibrium pH gradient electrophoresis gel. An unlabeled extract of A431 cells was dialyzed briefly, passed over a DE-52 cellulose column to enrich for p35 and p36 (calpactins) as described by Glenney (in press), and mixed with an immunoprecipitate of p36 (MAb D1/274) from [35 S]methionine-labeled A431 cells as shown in Fig. 3A. The sample was resolved by two-dimensional electrophoresis based on separation by charge in the first dimension (more basic proteins to the right side) and size (10% SDS-polyacrylamide gel) in the second by the method of Glenney (in press). The resulting gel was stained with Coomassie blue (a) and exposed to film for 18 h (b). The positions of p35 and p36, as determined by Glenney (in press), are indicated.

dimensions (Fig. 2B). Peptides were then individually eluted and subjected to phosphoamino acid analysis (11), pp36 from untreated or EGF-treated cells yielded four phosphoserine-containing peptides (peptides 1 through 4). Treatment with PDGF resulted in the appearance of two new phosphotyrosine-containing peptides (peptides 5 and 6). Treatment of the cells with the tumor promoter 12-

O-tetradecanoylphorbol-13-acetate (TPA; Sigma Chemical Co., St. Louis, Mo.) for the same period of time caused an eightfold increase in p36 phosphorylation on serine residue(s), due to a large increase in the phosphorylation of peptide 4, and a smaller, more variable increase in the phosphorylation of peptides 1 and 3 (Fig. 2A and B). Further experiments to identify this site of TPA-induced phosphor-

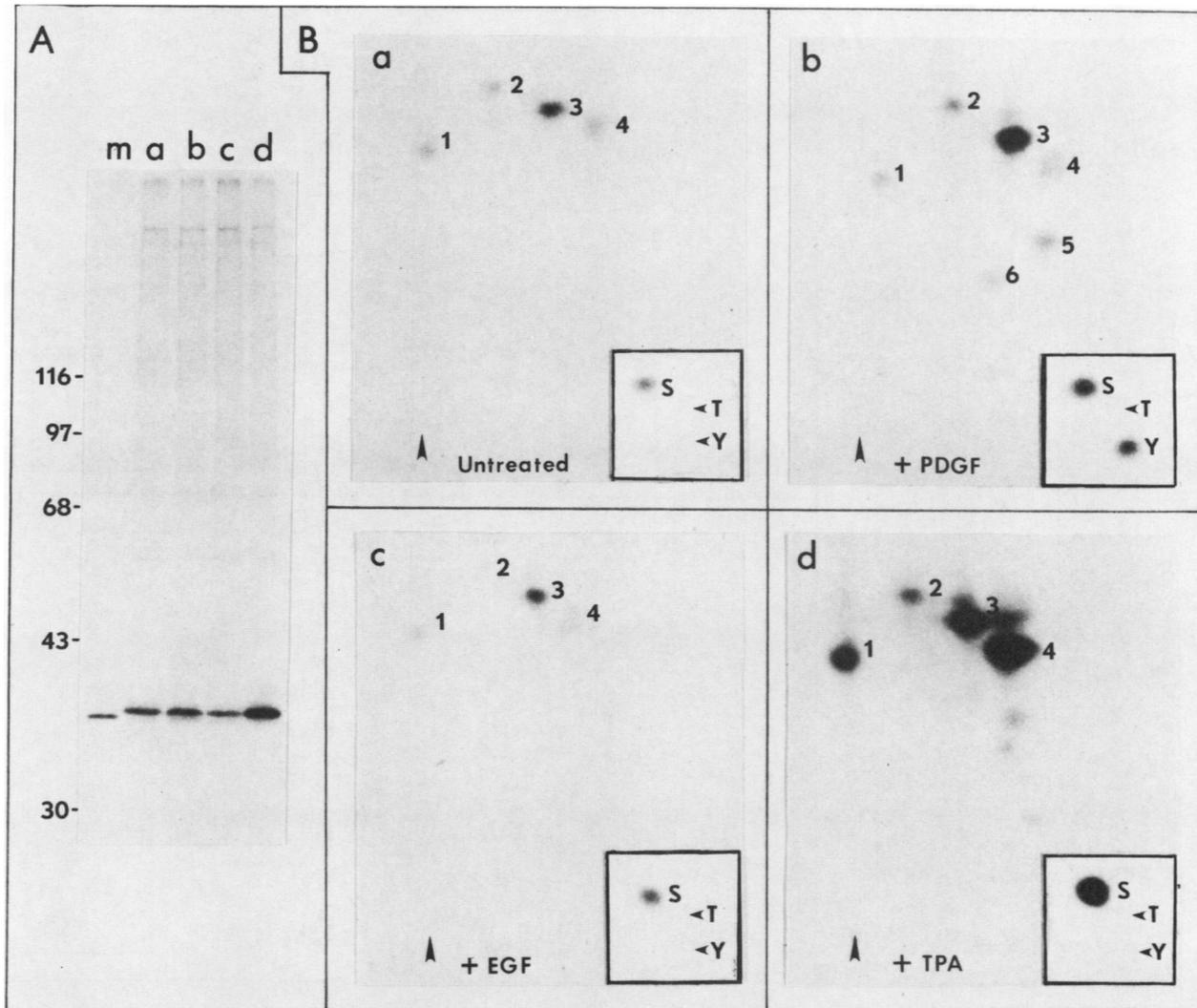


FIG. 2. Immunoprecipitation, tryptic peptide mapping, and phosphoamino acid analysis of phosphorylated p36 from AG1523 human fibroblasts. (A) Dishes (35 mm) of nearly confluent AG1523 cells were labeled for 18 h in 1 ml of either methionine- or phosphate-free Dulbecco minimal essential medium containing 4% dialyzed FBS and 150 μ Ci of [35 S]methionine or 1.5 mCi of carrier-free 32 P; (ICN Pharmaceuticals, Inc., Irvine, Calif.). Cells were left untreated or treated for 10 min with 25 ng of PDGF (36) per ml, 50 ng of EGF (40) per ml, 100 ng of TPA per ml, or 100 ng of 4- β -phorbol (Sigma) per ml. p36 was immunoprecipitated with MAb D1/274 and analyzed on a 10% SDS-polyacrylamide gel. Film exposure time was 18 h. Lanes: m, [35 S]methionine-labeled, untreated; a through d, 32 P-labeled and untreated (a), PDGF treated (b), EGF treated (c), and TPA treated (d). (B) Phosphorylated p36 was excised from the gel shown in panel A, subjected to tryptic digestion, and separated in two dimensions on cellulose thin-layer plates (100 μ m) by electrophoresis in the horizontal dimension at pH 1.9 for 25 min at 1 kV, followed by chromatography as previously described (32). In each case the origin is marked (arrowhead). pp36 tryptic peptide maps from cells treated with 4- β -phorbol and untreated cells were identical. Exposure time at -70° C to presensitized film with an intensifying screen was 7 days. The sizes of the samples (Cerenkov counts per minute) that were loaded onto thin-layer plates were as follows: a, 100; b, 280; c, 100; d, 740. In the insets, one-eighth of each sample eluted from the gel shown in panel A was subjected to phosphoamino acid analysis (11) before trypsin digestion. Abbreviations: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. Exposure time (at -70° C to presensitized film with an intensifying screen) was 6 days.

ylation and study the phosphorylation of p36 in vitro by protein kinase C are described in the preceding study (24).

To extend our study of the tyrosine phosphorylation of p36, we examined A431 human epidermoid tumor cells. These cells were chosen for two reasons. (i) A431 cells have a very large number of EGF receptors (17, 26), and EGF treatment results in the increased tyrosine phosphorylation of a number of proteins, including the EGF receptor itself, p81, and p36 (10, 16, 30). (ii) Unlike most human cells, permanent A431 cell lines transformed by a number of different tumor viruses are available, and it is therefore

possible to examine the phosphorylation of human p36 in response to virally encoded protein-tyrosine kinases.

p36 was immunoprecipitated from [35 S]methionine-labeled, nontransformed A431 cells, and from RSV-transformed and Snyder-Theilín feline sarcoma virus (ST-FeSV)-transformed A431 cells (Fig. 3A). All three cell types contained a large amount of p36 (about 0.4 to 0.8% total cell protein). As judged by tryptic peptide mapping, the proteins recognized by MAb D1/274 in these cells and in AG1523 cells were identical (data not shown). In 32 P-labeled A431 cells, pp36 was basally phosphorylated to a low level and con-

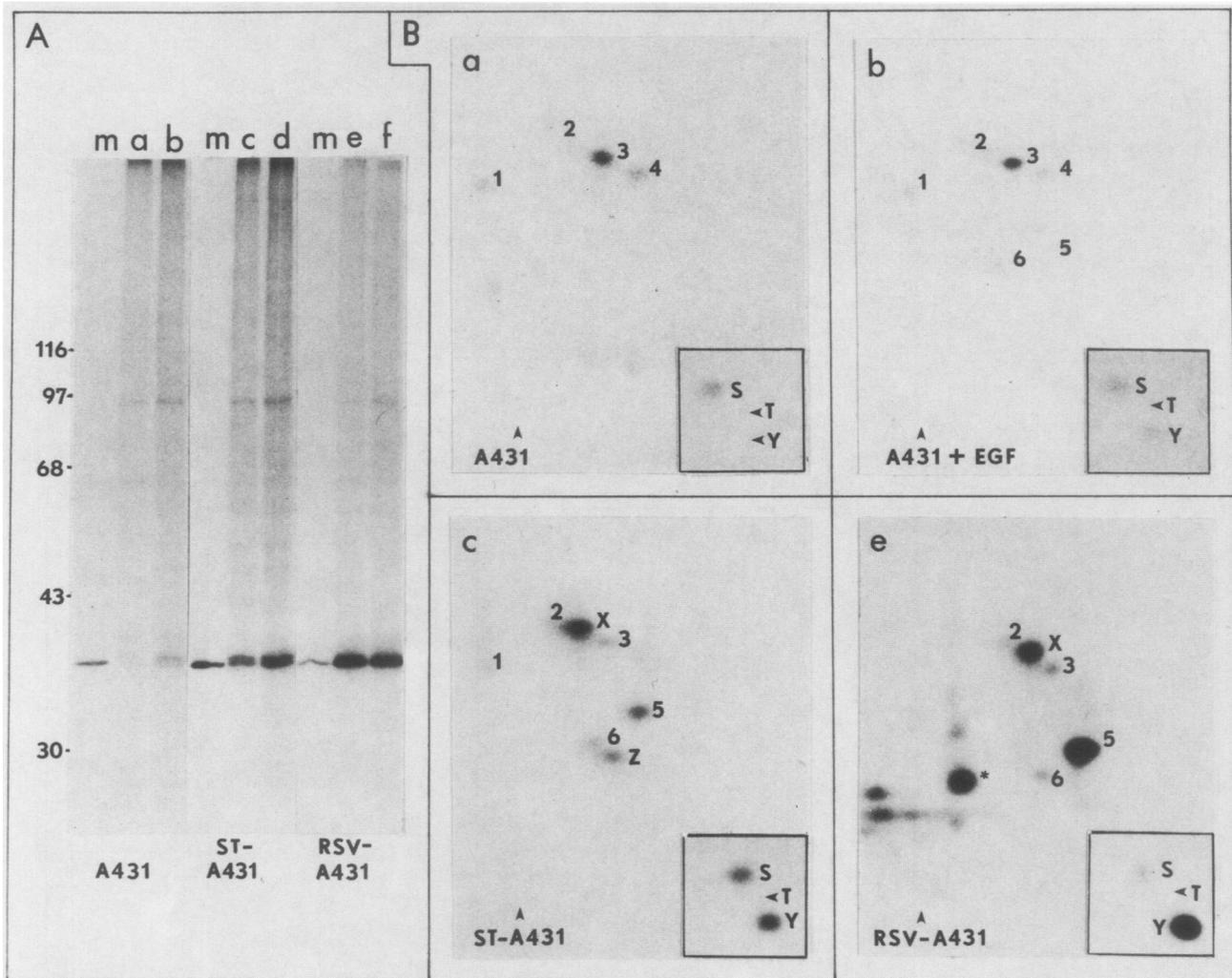


FIG. 3. Immunoprecipitation, tryptic peptide mapping, and phosphoamino acid analysis of p36 from A431 cells. A431 cells (subclone 8) were obtained from Gordon N. Gill, University of California at San Diego. RSV-transformed A431 (RSV-A431) cells and ST-FeSV-transformed A431 (ST-A431) cells, which express the P85^{gag-fes} and pp60^{v-src} protein-tyrosine kinases, respectively (27, 32), were from Edward M. Scolnick, Merck Sharp & Dohme, West Point, Pa. (10). (A) Dishes (35-mm) of nearly confluent A431 cells, ST-FeSV-transformed A431 cells, and RSV-transformed A431 cells labeled for 18 h with 1.5 mCi of ³²P; or 300 μ Ci of [³⁵S]methionine were treated for 10 min with 50 ng of EGF per ml before lysis as described in the legend to Fig. 2. p36 was immunoprecipitated with MAb D1/274 and resolved on a 10% SDS-polyacrylamide gel. Lanes: m, [³⁵S]methionine-labeled cells; a through f, ³²P-labeled cells; a, c, and e, untreated cells; b, d, and f, EGF-treated cells. Film exposure time was 2 days. (B) Phosphorylated p36 was excised from the gel shown in panel A, subjected to tryptic digestion, and separated in two dimensions as described in the legend to Fig. 2. Samples were loaded onto thin-layer plates, and samples sizes and exposure times (to presensitized film with intensifying screens at -70°C) were as follows: 100 cpm for 7 days (a), 100 cpm for 7 days (b), 200 cpm for 4 days (c), 450 cpm for 3 days (e). In the insets one-eighth of each sample eluted from the gel shown in panel A was subjected to phosphoamino acid analysis before trypsin digestion. Exposure time to presensitized film with an intensifying screen at -70°C was 6 days. Abbreviations are as described in the legend to Fig. 2.

tained the same tryptic phosphopeptides as pp36 from untreated AG1523 cells (Fig. 3B). A 10- or 40-min exposure of A431 cells to EGF before lysis resulted in a small (1.2- to 1.5-fold) increase in the level of p36 phosphorylation and the appearance of phosphotyrosine-containing peptides 5 and 6, previously identified in pp36 from PDGF-treated human fibroblasts (Fig. 2B). Since A431 cells possess about 6 to 20 times more EGF receptors than do human fibroblasts (4, 17, 26, 29), if there were only an equivalent response per receptor in fibroblasts treated with EGF, this response would be below the level of detection.

Our studies demonstrate for the first time that different growth factor treatments in unrelated cell types can result in

equivalent phosphorylation events of a common substrate protein.

Modulation of p36 phosphorylation in virally transformed A431 cells. Phosphorylated p36 immunoprecipitated from ST-FeSV-transformed A431 cells contained approximately equivalent quantities of phosphotyrosine and phosphoserine (Fig. 3B). Tryptic peptide maps of pp36 and subsequent phosphoamino acid analysis of individual phosphopeptides showed that, in these cells, the growth factor-responsive phosphotyrosine-containing peptides (peptides 5 and 6) were constitutively present, and in addition, two new phosphotyrosine-containing peptides were seen (peptides X and Z). pp36 from RSV-transformed A431 cells showed a pattern

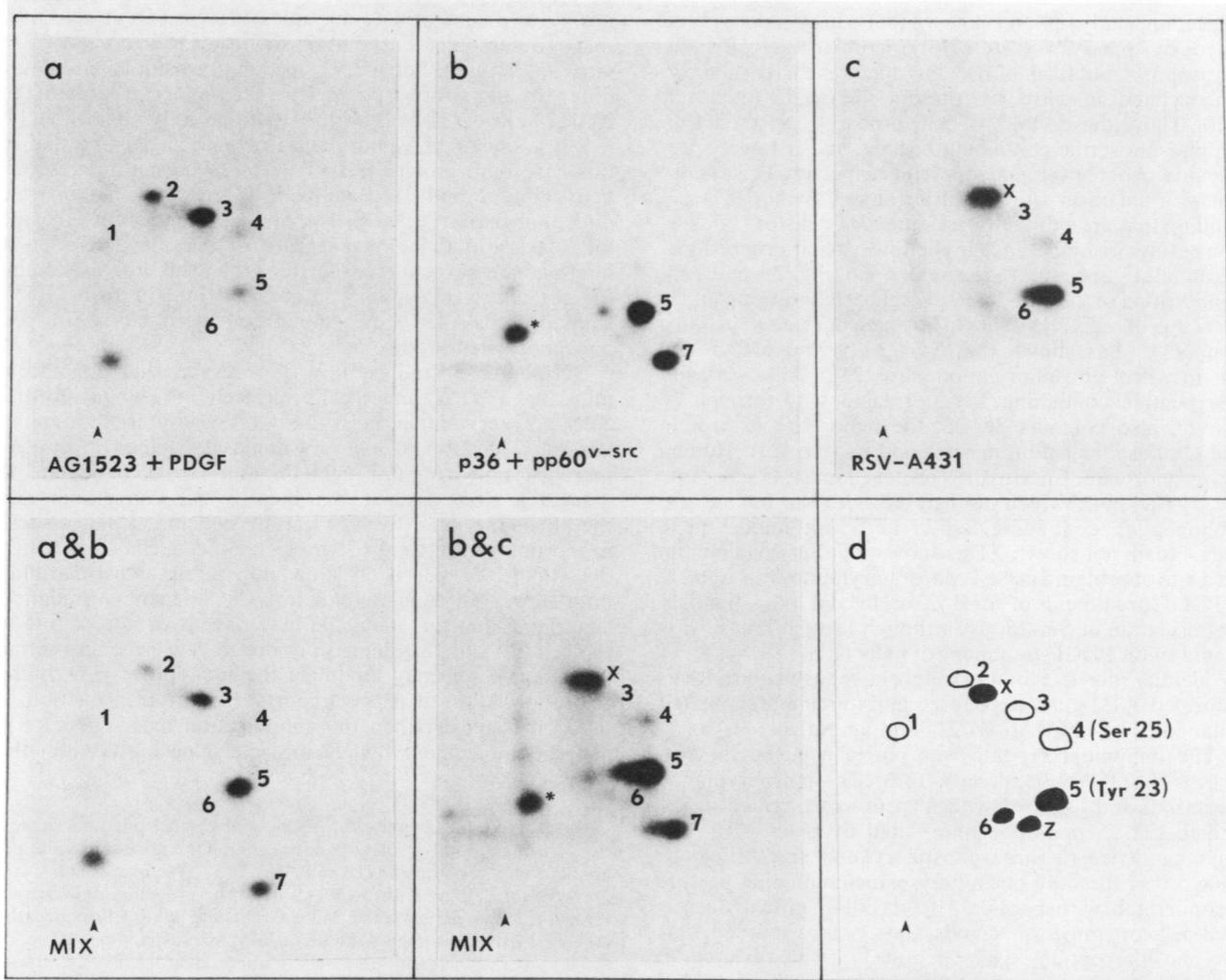


FIG. 4. In vitro phosphorylation of human p36 with pp60^{v-src}. (A) A 35-mm dish of nearly confluent RSV-transformed A431 cells was lysed in RIPA buffer, and p36 and pp60^{v-src} were coimmunoprecipitated with anti-human p36 MAb D1/274 and anti-pp60^{v-src} MAb 327 (33), respectively. The immunoprecipitates were washed twice in 20 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid) (pH 7.0)] and resuspended in 20 mM PIPES (pH 7.0), and the reaction was started by the addition of 10 mM MnCl₂ and 20 μCi of [γ -³²P]ATP (3,000 Ci/mmol; Amersham Corp.) to give a final reaction volume of 20 μl. After incubation at 30°C for 10 min, the reaction was terminated by the addition of 20 μl of 2× concentrated SDS-electrophoresis sample buffer and heating to 100°C for 2 min. The samples were then centrifuged (10,000 × *g*, 3 min) to sediment the *S. aureus*, and the supernatants were loaded onto a 10% SDS-polyacrylamide gel. p36 was excised, subjected to tryptic digestion, and separated in two dimensions as described in the legend to Fig. 2. In addition, samples from Fig. 2 (AG1523 cells treated with PDGF [a]) and Fig. 3 (RSV-transformed A431 cells [c]) were mapped separately and together with the in vitro-phosphorylated sample (b). A schematic diagram of the tryptic phosphopeptides of p36 is also shown (d). To determine the phosphoamino acid content of each spot, phosphopeptides were eluted and individually subjected to phosphoamino acid analysis; in panel d, open spots indicate phosphoserine-containing peptides, and closed spots indicate phosphotyrosine-containing peptides. Samples were loaded onto thin layer plates, and sample sizes and exposure times (to presensitized film with intensifying screens at -70°C) were as follows: 320 cpm for 2 days (a) b, 600 cpm for 2 days; 250 cpm for 3 days (c), 280 cpm each for 2 days; (a & b), 250 cpm each for 3 days (b & c).

of phosphorylation qualitatively similar to that of pp36 from ST-FeSV-transformed A431 cells (Fig. 3B). However, there was proportionately more phosphotyrosine and a difference in the relative amounts of phosphotyrosine-containing peptides; i.e., peptides Z and 6 were weaker while spots X and 5 were more intense. The presence of spot * was variable (Fig. 3 and 4). Analysis of this spot did not reveal any phosphorylated serine, threonine, or tyrosine residues, and its origin is unknown. Thus, transformation of A431 cells by either RSV or ST-FeSV resulted in at least one phosphotyrosine-containing peptide (peptide X) in addition to those found in growth factor-treated cells (peptides 5 and 6). Previously, Cooper and Hunter (6) found that pp36 from

both growth factor-treated and virally transformed A431 cells contained a single comigrating, phosphotyrosine-containing peptide, while Erikson et al. (16) reported that pp36 from EGF-treated cells and pp36 phosphorylated in vitro with pp60^{v-src} or pp60^{c-src} contained two comigrating peptides. In our study, a p36 tyrosine phosphorylation site unique to the virally transformed cells may have been detected because MAb D1/274 has a higher affinity than the polyvalent anti-p36 serum for pp36 phosphorylated at this site.

Phosphorylated forms of p36 were in some cases resolved as a doublet in 10% polyacrylamide gels (Fig. 2A and 3A). From in vitro and in vivo experiments presented here and in

the preceding study (24), it is known that p36 phosphorylated in vitro by pp60^{v-src} (see below) comigrates with the nonphosphorylated form of p36 and migrates faster than p36 phosphorylated in vitro by protein kinase C (data not shown). Thus, the doublet of p36 probably represents an upper phosphoserine-containing species and a lower, predominantly phosphotyrosine-containing species. This is consistent with the observations of Ralston and Bishop (37) and our finding that growth factor treatment of cells or transformation results in an increase in the lower band proportional to the amount of phosphotyrosine in pp36 (Fig. 2A and 3A).

Identification of the site of p36 phosphorylation by pp60^{v-src}. Sequencing of peptides from p36 phosphorylated in vitro with pp60^{v-src} has shown that Tyr-23 (bovine p36) is the major in vitro phosphorylation site (23). The predicted tryptic peptide containing Tyr-23 (residues 10 through 27 inclusive), also contains Ser-25, the major site of protein kinase C phosphorylation in vitro and in vivo (24). Human p36 phosphorylated in vitro by protein kinase C gave one major tryptic spot, which comigrated with the bovine p36 phosphopeptide containing Ser-25 and with human pp36 peptide 4 (data not shown). This shows that Ser-25 in human p36 is a site of protein kinase C phosphorylation. It is notable that PDGF treatment of AG1523 cells did not stimulate phosphorylation at Ser-25, even though protein kinase C is activated upon PDGF treatment of cells (1).

To identify the site of tyrosine phosphorylation, RSV-transformed A431 cells were lysed and immunoprecipitated simultaneously with MAb D1/274 and an anti-*src* MAb 327 (33). The immunoprecipitate was phosphorylated in vitro with [γ -³²P]ATP, resolved on a 10% SDS-polyacrylamide gel, excised, and digested with trypsin as described in the legend to Fig. 2. pp36 phosphorylated by pp60^{v-src} in this manner gave rise to three phosphorylated spots (Fig. 4). Peptide 5 was the only phosphotyrosine-containing peptide and comigrated with peptide 5 from either growth factor-treated cells or transformed cells. Spot *, as discussed, was not a phosphopeptide, while peptide 7, which varied in intensity between experiments, contained only phosphoserine. Since peptide 7 was found in immunoprecipitates of p36 incubated alone or with protein kinase C (data not shown), it is not specific to pp60^{v-src}. We conclude that there was only one major site of pp60^{v-src} phosphorylation in human p36 in vitro and this corresponded to Tyr-23. Therefore, Tyr-23 was a substrate for both virally encoded and growth factor receptor protein-tyrosine kinases. It is also noteworthy that phosphorylation of the identical peptide on Tyr-23 (peptide 5) or Ser-23 (peptide 4) resulted in a peptide with a markedly different chromatographic mobility.

Conclusions. The anti-human p36 MAbs have enabled us to carry out an extensive analysis of the phosphorylation of p36 in human cells under a variety of conditions. There are several explanations for why different patterns of phosphorylation of p36 on tyrosine residues were observed under the various experimental conditions. First, it may be that the two oncogenic protein-tyrosine kinases and the growth factor receptors have differential access to p36 in vivo. p36 itself is located on the inner side of the plasma membrane in association with the underlying cytoskeleton. The growth factor receptors are integral cell membrane proteins. P85^{gag-fes} and pp60^{v-src} are both myristylated at their N termini (3, 42, 43), but whereas pp60^{v-src} is predominantly associated with the plasma and perinuclear membranes (12, 38), P85^{gag-fes} is found in the cytosol as well as in association with membranes (39). Similarly, the finding that pp60^{v-src} only phosphorylated one tyrosine residue in vitro but ap-

peared to phosphorylate multiples sites in p36 in vivo may reflect a difference in the accessibility of p36 to pp60^{v-src} in vitro and in vivo. Second, the protein-tyrosine kinases may differ in substrate specificity. However, treatment of AG1523 and A431 cells with PDGF and EGF, respectively, led to a similar phosphorylation of p36, suggesting that in this case both growth factor receptors have similar specificity (Fig. 2 and 3). Finally, it is possible that in vivo phosphorylation of p36 by oncogenic protein-tyrosine kinases or growth factor receptors involves the activation of intermediate protein-tyrosine kinases. This could account for the extra phosphorylation sites in p36 from RSV-transformed A431 cells compared with those in p36 phosphorylated in vitro by pp60^{v-src}.

During these studies, it was observed that pp36 from untreated or TPA-treated cells migrated more slowly on 10% SDS-polyacrylamide gels than did the [³⁵S]methionine-labeled (Fig. 2A and 3A) or Coomassie-stained forms. As there was only a minor [³⁵S]methionine-labeled band, and no detectable Coomassie-stained band, which comigrated with the phosphorylated form, it can be concluded that a small proportion of the total p36 molecules were phosphorylated (less than 5%). p36 is an abundant protein and it can bind both actin and phospholipids in a Ca²⁺-dependent manner, suggesting that this molecule may have a structural role in the cell (19, 20, 22; Glenney, in press). Tyrosine and serine phosphorylation may modulate the function of p36. If the phosphorylation of p36 is important in the transduction of mitogenic signals, then this modification must occur on a subset of molecules which lie in strategic positions within the cell.

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