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Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases

(pp60^{src}/anti-phosphotyrosine/ELISA/transformation)

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ABSTRACT **Cellular transformation by oncogenic retroviruses encoding protein tyrosine kinases coincides with the tyrosine-specific phosphorylation of multiple protein substrates. Previous studies have shown that tyrosine phosphorylation of a protein of 120 kDa, p120, correlated with *src* transformation in chicken embryo fibroblasts. Additionally, we previously identified two phosphotyrosine-containing cellular proteins, p130 and p110, that formed stable complexes with activated variants of pp60^{src}, the *src*-encoded tyrosine kinase. To study transformation-relevant tyrosine kinase substrates, we have generated monoclonal antibodies to individual tyrosine phosphoproteins, including p130, p120, p110, and five additional phosphoproteins (p210, p125, p118, p85, and p185/p64). These antibodies detected several of the same tyrosine phosphoproteins in chicken embryo fibroblasts transformed by avian retroviruses Y73 and CT10, encoding the *yes* and *crk* oncogenes, respectively. Protein substrates in mouse, rat, hamster, and human cells overexpressing activated variants of chicken pp60^{src} were also detected by several of the monoclonal antibodies.**

The role of tyrosine phosphorylation of cellular proteins by oncogene-encoded tyrosine kinases during transformation is largely unclear. Multiple protein substrates are phosphorylated on tyrosine in response to various cell stimuli, including growth factor activation (1–5), agonist stimulation of secretion (6), platelet activation (7–9), cell cycle changes (10, 11), and cell transformation induced by oncogene-encoded tyrosine kinases (12–21). Although the majority of these cellular protein substrates have not been identified, recent studies have demonstrated the interactions of known tyrosine phosphoproteins thought to be involved in signal-transduction pathways (22). Direct identification of the individual proteins may provide information regarding their activities during cell growth processes and perhaps lead to an understanding of the relevance of tyrosine phosphorylation of proteins *in vivo*.

The study of tyrosine-phosphorylated cellular proteins has recently relied on antibodies to phosphotyrosine (anti-*P*-Tyr) (13, 15, 16, 23, 24). Immunoblot analysis of cell proteins with anti-*P*-Tyr has revealed previously undetected *P*-Tyr-containing proteins in both normal and transformed cells. Specifically, expression of the oncogene-encoded tyrosine kinase pp60^{src} of Rous sarcoma virus (RSV) leads to the tyrosine phosphorylation of 15–20 different cellular substrates in several cell systems (13, 16, 17, 19, 20).

We and others have demonstrated that one phosphoprotein of 120 kDa, p120, may be relevant to transformation of chicken embryo fibroblasts (CEFs) by activated pp60^{src} (17, 20). A mutation of the normal c-*src* gene that changes the C-terminal tyrosine residue (Tyr-527) to phenylalanine was sufficient to induce both cell transformation (25–29) and tyrosine phosphorylation of p120 (17, 20). However, an additional mutation at the N-terminal glycine residue at position 2 (the site of myristoylation for membrane association) in the activated *src* protein (pp60^{527F} → pp60$^{2A/527F}$) ablated both p120 phosphorylation and transforming activity (17, 20). Moreover, two additional tyrosine phosphoproteins of 130 and 110 kDa were observed in immunocomplexes of activated pp60^{src} proteins by anti-*P*-Tyr immunoblotting (12). Analysis of the p130/p110/pp60^{src} complex has shown that two parameters are required for stable protein interaction: (*i*) activation of the tyrosine kinase activity of pp60^{src} *in vivo* and (*ii*) an intact *src* homology domain (30) in pp60^{src}, an N-terminal region with sequence similarity to the *crk* oncogene product, the *ras* GTPase-activating protein, α-spectrin, and phospholipase C-γ (31, 32). These studies indicate that tyrosine phosphorylation of several cellular proteins may be important during cellular transformation by pp60^{src}.

To study individual tyrosine phosphoprotein substrates, we have generated monoclonal antibodies (mAbs) to p130, p120, and p110, and five other proteins that are tyrosine-phosphorylated in CEFs expressing activated variants of pp60^{src}. We present the derivation and characterization of these mAbs with respect to their use in the study of potentially relevant substrates of several tyrosine kinases in different cell systems.

MATERIALS AND METHODS

Cells, Viruses, and Plasmids. Primary CEFs were prepared from gs-negative embryos (SPAFAS, Norwich, CT) (20). pRL-527F and pRL-2A/527F, plasmids bearing chicken c-*src* gene mutants in a nonpermuted RSV clone, were constructed and transfected as described (20). NIH 3T3 (mouse fibroblast line), Rat-1 (rat fibroblast line), HOS (human osteosarcoma cell line), 10W (asbestos-transformed Syrian hamster embryo cell line; ref. 33), and their v-*src*-transformed cognate cell lines were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

Immunoprecipitations and Immunoaffinity Chromatography. Immunoprecipitations and immunoaffinity purification of tyrosine phosphoproteins from CEFs with affinity-purified rabbit anti-*P*-Tyr were performed as described (34).

Immunizations and Fusion Protocol. Immunoaffinity-purified tyrosine phosphoproteins derived from CEFs expressing pp60^{527F} or pp60$^{2A/527F}$ (5 μg each) were combined and emulsified in 350 μl of 50% complete Freund's adjuvant and injected both subcutaneously and intraperitoneally into individual female A/J strain mice (The Jackson Laboratory) for the initial immunization. At 1-month intervals after immunization, the mice received booster injections of the equivalent

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Abbreviations: CEF, chicken embryo fibroblast; mAb, monoclonal antibody; anti-*P*-Tyr, antibody(ies) to phosphotyrosine; HRP, horseradish peroxidase; RSV, Rous sarcoma virus.

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antigen used for primary immunizations. After an additional month, 30 μg of mixed antigen in phosphate-buffered saline was injected intraperitoneally either 4 days prior to fusion or 8, 6, and 4 days (10 μg/day) prior to fusion. The spleen cells were fused with the SP2/0 myeloma cell line as described (35, 36). The cell fusions were dispersed into five to eight 96-well plates (containing 100% of the cells), screened for growth 1–7 days after plating, and then screened for mAb production by ELISA starting on day 8. ELISA/immunoprecipitation-positive hybridomas were subcloned twice by limiting dilution, ascites were generated in CAF/1J mice, and mAbs were purified by protein A-Sepharose affinity chromatography.

ELISA. The direct ELISA was performed by coating Immulon II (Dynatech) 96-well plates with 100 ng of mixed purified tyrosine phosphoproteins per well for 16 hr at 4°C. After the plates were washed with TBS/V (10 mM Tris, pH 7.3/150 mM NaCl/10 μM sodium orthovanadate) and blocked with 0.05% Tween 20 in TBS/V, hybridoma culture supernatants (100 μl) were incubated for 3 hr at 4°C followed by addition of horseradish peroxidase (HRP)-conjugated second antibody for 3 hr. Color reactions were developed with *o*-phenylenediamine as substrate (37). For the indirect assay, plates were coated with 100 μl of rabbit anti-mouse IgG (Jackson ImmunoResearch) at 4 μg/ml and incubated for 16 hr at 4°C. Culture supernatants were added as described above, followed by 100 μl of mixed cell lysate (≈300 μg) from CEFs expressing $pp60^{527}$ or $pp60^{2A/527}$. Biotinylated, affinity-purified rabbit anti-*P*-Tyr (1:2000 dilution) were added and incubated for 3 hr at 4°C. Avidin-conjugated HRP was then added and color reactions were developed as described above. Determination of positive hybridoma cultures was based on signals >0.3 A_{492} unit above background.

Immunoblotting. Immunoprecipitated proteins were subjected to SDS/8% PAGE (38) and transferred to nitrocellulose (BA-83, Schleicher & Schuell). After incubation in 4% bovine serum albumin in TN buffer (50 mM Tris, pH 7.5/150 mM NaCl) at 37°C for 4 hr, the filters were probed with affinity-purified rabbit anti-*P*-Tyr as described (12, 13, 16, 20, 34). For immunoblotting assays with mAbs, the albumin/TN blocking solution was supplemented with 5% nonfat dry milk (33), and the filters were incubated with mAb at 2 μg/ml for 16 hr at 4°C, then incubated with ^{125}I-labeled sheep anti-mouse IgG (Amersham) at 1 μCi/ml (1 μCi = 37 kBq), washed, and exposed to Kodak X-RP film at −70°C with intensifying screens.

RESULTS

Generation and Isolation of mAbs to Individual Protein Substrates of $pp60^{src}$. We previously reported the purification of tyrosine-phosphorylated cellular proteins by immunoaffinity chromatography with anti-*P*-Tyr (34). This procedure was used to prepare tyrosine phosphoproteins from CEFs infected with variants of RSV (527F and 2A/527F) encoding activated forms of the c-*src* protooncogene protein, $pp60^{c\text{-}src}$ (20). RSV-527F contains a mutation in the *src* gene altering the C-terminal Tyr-527 to phenylalanine. This mutation activates both the tyrosine kinase and the transforming activity of $pp60^{src}$ (25–29). RSV-2A/527F contains an additional mutation altering the N-terminal glycine residue at position 2 to alanine. A mutation at this site in $pp60^{src}$ blocks myristoylation and prevents association with cellular membranes (17, 19, 20). The expression of these activated variants of $pp60^{src}$ induced the tyrosine phosphorylation of 15–20 cellular proteins (16, 17, 19, 20), many of which have been purified 3000-fold with anti-*P*-Tyr immunoaffinity resins (34). Since the expression of these two c-*src* variants results in both common and different tyrosine phosphorylations, antigens purified from each cell type were combined and used in the immunization procedures.

Four A/J strain mice were immunized with mixed *P*-Tyr-containing antigens by both subcutaneous and intraperitoneal injection. To assess the immunogenicity of the various phosphoproteins, the mice were bled 10 days after immunization, and the sera were assayed for antibodies to tyrosine phosphoproteins by immunoprecipitation and anti-*P*-Tyr immunoblotting. Immune responses to several proteins were detected in two different mice, and increased titers to individual proteins were observed upon successive injections with antigen (data not shown).

To identify mAbs to tyrosine phosphoproteins and to distinguish mAbs to non-tyrosine-phosphorylated contaminating proteins in the immunoaffinity-purified antigen mixtures, two different ELISAs were developed. The direct ELISA (Fig. 1*A*) relied on the use of the immunoaffinity-purified *P*-Tyr-containing protein mixture as the source of antigen. The mAb, bound to its respective antigen, was detected by a rabbit anti-mouse immunoglobulin conjugated to HRP, followed by development of a color reaction. This assay detected mAbs to highly abundant *P*-Tyr-containing antigens and to copurified non-tyrosine-phosphorylated proteins in the mixture. The indirect ELISA (Fig. 1*B*) relied on cell lysates from CEFs expressing $pp60^{527F}$ and $pp60^{2A/527F}$ as the source of tyrosine-phosphorylated antigen. In this assay, the mAb contained in individual hybridoma supernatants was captured by an unlabeled rabbit anti-mouse immunoglobulin. Cell lysates were then incubated to allow binding of antigen to the mAb. After washing, biotinylated affinity-purified rabbit anti-*P*-Tyr was added. This step required that the mAb-bound antigen contained an immunoreactive *P*-Tyr residue that was displaced from the mAb-binding epitope.

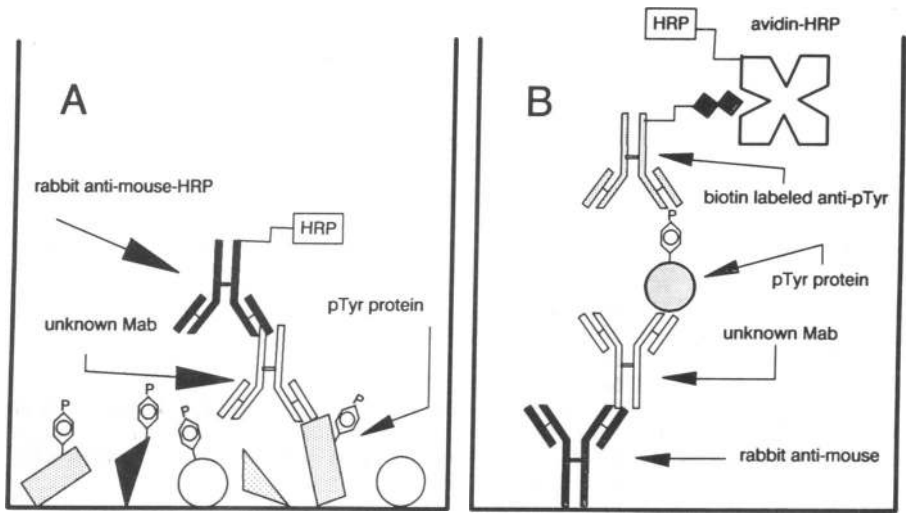

FIG. 1. ELISAs for mAbs to tyrosine phosphoproteins. (*A*) The direct assay was performed with immunoaffinity-purified tyrosine phosphoproteins as antigen. mAbs were detected with HRP-conjugated rabbit antibodies to mouse immunoglobulin. (*B*) In the indirect assay the source of antigen was cell lysates from CEFs expressing activated *src* variants. Detection of mAbs was performed with biotinylated-rabbit anti-*P*-Tyr binding to the same antigen as mAb, followed by reaction with avidin-conjugated HRP.

The positive signal was then detected by avidin-HRP conjugates and a color reaction. The indirect assay was specific for antigens that contained a *P*-Tyr residue, and was more sensitive than the direct ELISA in detecting mAbs to minor antigens.

Spleen cells from two mice with serum titers to several antigens were fused with SP2/0 myeloma cells (35, 36), and the resulting clones were screened by both the direct and the indirect ELISA. In fusion I, 426 hybridomas resulted from the plating of fused cells into 768 wells (55%). ELISA-positive clones were detected in 80/426 wells (19%), 16 of which were positive by subsequent immunoprecipitation/immunoblotting assays. In fusion II, of 360 hybridomas that grew in 480 wells plated (75%), 171 were positive by ELISA (47%), yielding 16 mAbs positive by immunoprecipitation/immunoblotting assays (see Table 1 for details). mAbs directed to p210, p130, p85, p185/p64, and *P*-Tyr were readily detected by both direct and indirect ELISAs, while mAbs to p125, p120, p118, and p110 were detected only in the indirect ELISA.

Characterization of Individual mAbs. mAbs to eight different tyrosine phosphoproteins, and mAbs directed to *P*-Tyr, were identified and subcloned by limiting dilution. Fig. 2 shows the eight proteins immunoprecipitated by their respective mAbs and detected by anti-*P*-Tyr immunoblotting. The proteins were designated by their mobility in SDS/polyacrylamide gels as follows: p210 (lane 1), p130 (lane 2), p125 (lane 3), p120 (lane 4), p118 (lane 5), p110 (lane 6), p85 (lane 7), and p185/p64 (lane 8). For comparison, activated $pp60^{src}$ was immunoprecipitated (lane 9) with the previously generated mAb EC10 (39), showing the coimmunoprecipitation of p130 and p110 (12). Immunoprecipitation of the entire panel of proteins was achieved with a mAb generated to *P*-Tyr (lane 10). Although most of the mAbs immunoprecipitated a single protein species, three of the mAbs appeared to have complex specificities. mAb to p130 immunoprecipitated two additional unidentified proteins that were not detected by direct immunoblotting (data not shown). In addition, mAbs to p85 immunoprecipitated a triplet of proteins. Subsequent analysis using different anti-p85 mAbs exhibiting either chicken-specific reactivity or species crossreactivity showed that all three p85 bands were immunologically related (data not shown). mAbs to p185/p64 were directed to a protein of 185 kDa that was not phosphorylated in CEFs expressing $pp60^{src}$ but was coimmunoprecipitated with a tyrosine-phosphorylated protein of 64 kDa. All of the proteins immunoprecipitated by the individual mAbs contained *P*-Tyr as determined by phospho amino acid analysis of ^{32}P-labeled proteins (data not shown).

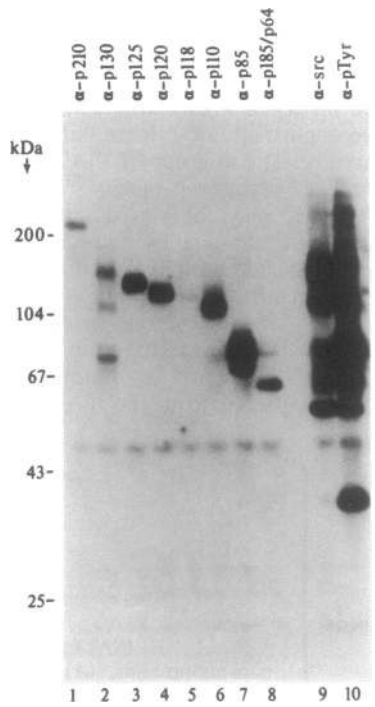

FIG. 2. Immunoprecipitation of *P*-Tyr-containing proteins with individual mAbs. mAbs were used to immunoprecipitate tyrosine phosphoproteins from 400 μg of a mixture of cell lysates from CEFs infected with viruses expressing $pp60^{527F}$ or $pp60^{2A/527F}$. Immunoprecipitated proteins were detected by anti-*P*-Tyr immunoblotting. mAbs used for immunoprecipitation were 3C4 (lane 1), 3D1 (lane 2), 2A7 (lane 3), 2B12 (lane 4), 1E12 (lane 5), 4C3 (lane 6), 3B12 (lane 7), 4H6 (lane 8), EC10 (lane 9), and 6G9 (lane 10). The bands at 50 kDa and ≈25 kDa are IgG heavy and light chains, respectively. α, Anti.

Characterization of the mAbs, including the molecular masses of the proteins they recognize, their reactivity in immunoprecipitation and immunoblot assays, their isotype, and their species crossreactivities, is summarized in Table 1. All of the mAbs immunoprecipitated their respective antigens, although several mAbs were unreactive in immunoblot

Table 1. Characterization of mAbs

Antigen recognized*	mAb	IP†	IB‡	Isotype	Cross-reactivity§
		Fusion I			
p130	3D1	+	+	IgM	+
	4F4	+	+	IgM	NT
	5F9	+	NT	IgM	NT
p125	2A7	+	−	IgG1	+
p120	2B12	+	+	IgG1	+
p118	1E12	+	−	IgG1	−
p85	1H3	+	+	IgG1	−
	3A7	+	NT	NT	NT
	3B12	+	+	IgG1	−
	5A7	+	NT	IgG1	NT
	6E12	+	NT	IgG2b	NT
p185/p64	4H6	+	+	IgG1	−
P-Tyr	3C10	+	+	IgG2a	+
	6C3	+	+	IgG1	+
	6G9	+	+	IgG2a	+
	8C4	+	+	IgG1	+
		Fusion II			
p210	1G5	+	NT	IgM	NT
	2F6	+	NT	IgG1	NT
	3C4	+	+	IgG1	NT
	5B9	+	NT	IgG1	NT
p110	4C3	+	+	IgG2b	±
p85	3D9	+	−	IgG1	+
	1H1	+	+	IgG1	+
	4F11	+	+	IgG1	+
p185/p64	4G8	+	+	IgG1	±
	4E10	+	+	IgG1	±
	2C5	+	+	IgM	NT
	5H10	+	+	IgG1	+
P-Tyr	1D6	+	+	IgM	+
	1D10	+	+	IgG3	+
	5E3	+	+	IgM	+
	5F11	+	+	IgG3	+

NT, not tested.

*Molecular masses of proteins (e.g., p130, 130 kDa) were determined by mobility in SDS/polyacrylamide gels.

†Immunoprecipitation reactivity.

‡Immunoblot reactivity.

§Crossreactivity of mAbs in immunoprecipitation assays with proteins from CEFs and four mammalian cell lines: NIH 3T3 (mouse), Rat-1 (rat), 10W (hamster), and HOS-1 (human). +, Crossreactivity with CEFs and all four cell lines; −, no crossreactivity; ±, weak crossreactivity.

assays. Interestingly, fusion of spleen cells from two different immunized mice yielded a distinct repertoire of mAbs to the antigen mixture used for immunization. Many of the mAbs crossreacted with the same proteins in mouse, rat, hamster, and human cells in immunoprecipitation assays; however, several were specific for chicken proteins, demonstrating the presence of conserved and nonconserved antigenic determinants. Finally, the predominant isotype was IgG1, although several mAbs were of the IgG2, IgG3, or IgM isotype.

To determine whether the mAbs to individual tyrosine phosphoproteins were directed to polypeptide sequences or to *P*-Tyr, phospho amino acids were used to block the immunoprecipitation of the individual proteins. mAbs to four individual tyrosine phosphoproteins were not inhibited by *P*-Tyr or phosphoserine (Fig. 3*B*). In addition, preincubation with *P*-Tyr did not block the immunoprecipitation with mAbs to p210, p118, p110, or p185/p64 (data not shown). However, in control experiments (Fig. 3*A*), the proteins immunoprecipitated by an anti-*P*-Tyr mAb (lane 1) were not detected after immunoprecipitation using mAb preincubated with *P*-Tyr (lane 2) or phenyl phosphate (lane 5), whereas neither phosphoserine nor phosphothreonine inhibited the immunoprecipitation (lanes 3 and 4). None of the phospho amino acids abrogated the immunoprecipitation of $pp60^{src}$ or the two phosphoproteins associated with activated $pp60^{src}$ (lanes 6–10). These data indicate that the mAbs were directed to determinants other than *P*-Tyr. Analysis of immunoprecipitates from cells labeled with [^{35}S]methionine and $^{32}P_i$ revealed that each mAb immunoprecipitated its respective protein, although several mAbs coimmunoprecipitated additional cell proteins. The nature of these protein complexes remains to be determined.

Tyrosine Phosphorylation of Common Substrates in CEFs Expressing v-*src*, v-*yes*, or v-*crk*. Acute type-C avian retroviruses contain transforming oncogenes, many of which encode tyrosine kinases. In addition to RSV, which encodes $pp60^{v\text{-}src}$, the Y73 retrovirus encodes the tyrosine kinase $pp90^{gag\text{-}yes}$ (14), and CT10 expresses an oncogene product without intrinsic tyrosine kinase activity, $p47^{gag\text{-}crk}$, although infection of CEFs with CT10 results in the novel tyrosine phosphorylation of several cellular proteins (32). To determine whether these viruses induced the tyrosine phosphorylation of common cellular protein substrates, p130, p125, p120, p110, and p85 were immunoprecipitated with their respective mAbs from CEFs infected with Y73, CT10, or RSV. Anti-*P*-Tyr immunoblotting of the immunoprecipitates showed that p130, p125, and p110 were all phosphorylated on tyrosine to different levels in CEFs expressing the three different viruses (Fig. 4). Increased p125 phosphorylation was assessed by a shift in mobility. In contrast, only the tyrosine kinases $pp60^{src}$ and $pp90^{gag\text{-}yes}$ phosphorylated p120 and p85 on tyrosine. Thus, although these protein substrates were initially identified in CEFs transformed by activated forms of $pp60^{src}$, they were also targets of at least one other tyrosine kinase.

DISCUSSION

The generation of anti-*P*-Tyr has facilitated the study of cellular protein substrates of tyrosine kinases (13, 15, 16, 23, 24). However, analysis of individual proteins is limited with these antibodies in systems with multiple tyrosine phosphoproteins. Here we report the generation of mAbs to individual tyrosine-phosphorylated cellular substrates of the tyrosine kinase $pp60^{src}$. Hybridomas producing mAbs to eight different tyrosine phosphoproteins, p210, p130, p125, p120, p118, p110, p85, and p185/p64, were derived from two separate fusions of spleen cells from mice immunized with immunoaffinity-purified tyrosine phosphoproteins from CEFs expressing activated forms of $pp60^{src}$. These mAbs detected the same proteins in both avian and mammalian cells and were directed to protein targets of several tyrosine kinases. In addition, we have also generated mAbs to *P*-Tyr by immunization with cellular substrates of tyrosine kinases.

In CEFs expressing the v-*yes* oncogene, p85 and, to a lesser extent, p120 and p110 were phosphorylated on tyrosine. Although the level of oncogene expression appeared to be comparable (data not shown), the level of tyrosine phosphorylation of these proteins was reduced relative to that observed in v-*src*-transformed CEFs. This might be due to differences in subcellular localization of the activated kinase or differences in substrate affinity and/or specificity. Tyro-

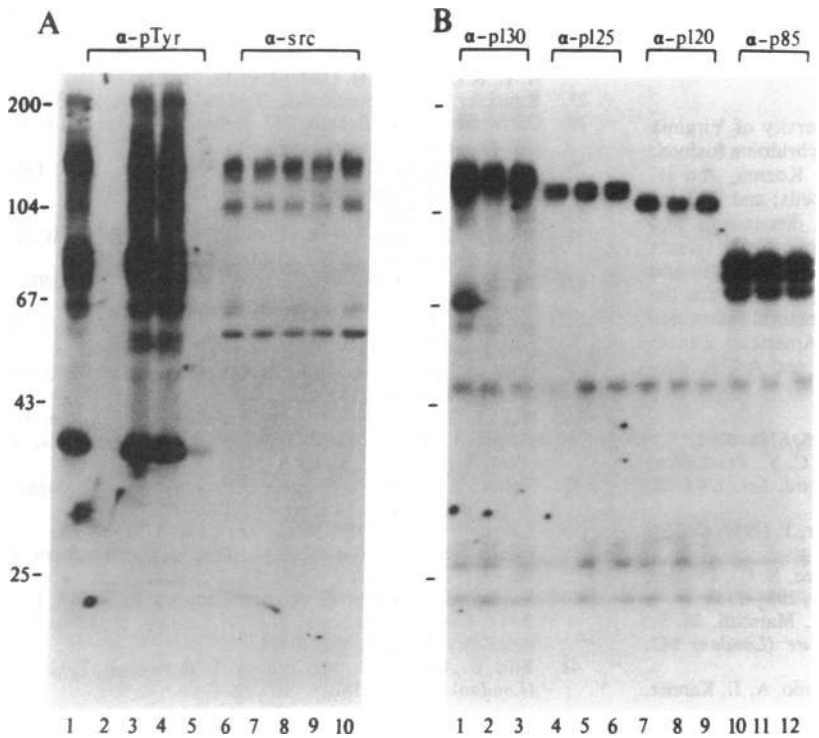

FIG. 3. Phospho amino acid inhibition assay. Immunoprecipitation assays as described in Fig. 2 were performed with anti-*P*-Tyr mAb (lanes 1–5) and anti-src mAb EC10 (lanes 6–10) (*A*) or with mAbs 3D1 (lanes 1–3), 2A7 (lanes 4–6), 2B12 (lanes 7–9), and 1H3 (lanes 10–12) (*B*). Phospho amino acids (40 mM) were preincubated with antibodies prior to immunoprecipitation. In *A*, phospho amino acids were absent (lanes 1 and 6), or *P*-Tyr (lanes 2 and 7), phosphoserine (lanes 3 and 8), phosphothreonine (lanes 4 and 9), or phenyl phosphate (lanes 5 and 10) was added. In *B*, phospho amino acids were absent (lanes 1, 4, 7, and 10), or *P*-Tyr (lanes 2, 5, 8, and 11) or phosphoserine (lanes 3, 6, 9, and 12) was included. Immunoprecipitated proteins were detected by anti-*P*-Tyr immunoblotting.

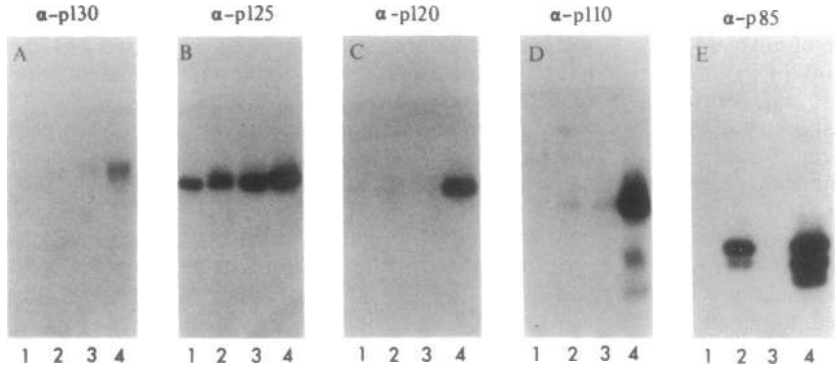

FIG. 4. Common substrates phosphorylated in CEFs expressing v-*src*, v-*yes*, or v-*crk*. Immunoprecipitations were performed as described in Fig. 2, followed by anti-*P*-Tyr immunoblotting. Uninfected CEFs (lanes 1) or CEFs infected with Y73 (lanes 2), CT10 (lanes 3), or RSV (lanes 4) were analyzed by immunoprecipitation with mAbs 3D1 (*A*), 2A7 (*B*), 2B12 (*C*), 4C3 (*D*), and 3B12 (*E*).

sine phosphorylation of cellular proteins has also been observed in mitogenic signal-transduction pathways (1–5, 22), during cell cycle changes (10, 11), during cell secretion upon agonist stimulation (6), and during platelet activation (7–9). These diverse cellular processes may include common cellular protein substrates that become phosphorylated on tyrosine in response to different cell stimuli.

The identities of the proteins detected by the mAbs remain unknown. However, we have eliminated several possibilities by assaying immunocomplexes directly for enzymatic activity or crossreactivity with available antisera to known proteins. None of the mAbs immunoprecipitated type I phosphatidylinositol kinase activity (40), except for the anti-*P*-Tyr mAbs (data not shown). In immunoblot assays, immunoprecipitates of p85 were not detected by antibodies to protein kinase C and immunoprecipitates of protein kinase C were not detected by mAbs to p85; immunoprecipitates of p110 were not detected by antibodies to chicken α-actinin, and vice versa; immunoprecipitates of p120 were not detected by antibodies to the fibronectin receptor, and vice versa; and immunoprecipitates of the p185/p64 complex were not detected with antibodies to GTPase-activating protein (41), and vice versa (data not shown). It is possible that one or more of the proteins recognized by our mAbs may be involved in receptor complexes similar to those reported between the platelet-derived growth factor β receptor and Raf-1 (22). Recently, methods similar to those described here were used to generate mAbs to individual CEF-derived tyrosine phosphoproteins (21), revealing a different repertoire of mAbs. It is unclear whether our mAbs are directed to any of the same protein substrates as those reported (21).

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