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Monoclonal antibodies to phosphotyrosine

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Phosphotyrosine coupled to KLH, BSA, and OVA was used for the production and screening of antibodies to phosphotyrosine. 800 hybridomas secreting antibodies that bound to phosphotyrosine were detected by ELISA. The most reactive 100 of these 800 were tested subsequently for their ability to bind phosphotyrosine-containing proteins on Western blots. Eight stable hybridoma cell lines were selected for further study, cloned by limiting dilution, and grown as ascites. These antibodies were purified by three different methods, and it was found that affinity chromatography on phosphotyramine-affigel provided the most rapid and effective method. Many phosphotyrosine-containing proteins were detected by using these antibodies in Western blotting and immunoaffinity purification procedures. Binding of anti-phosphotyrosine antibody could be competed by phosphotyrosine or phenylphosphate but not by phosphoserine, phosphothreonine, or free phosphate. These antibodies should be useful for the identification and purification of proteins phosphorylated on tyrosine residues in transformed and growth factor-treated cells.

Key words: Oncogene; Kinase; ELISA

Introduction

Many growth factor receptors and oncogene products are protein kinases that phosphorylate tyrosine residues (Hunter, 1987). In order to characterize the substrates of these kinases that may be involved in various aspects of growth regulation and oncogenesis, one needs methods to identify and isolate these cellular proteins. A number of studies have utilized the relative resistance of the phosphotyrosine linkage toward base hydrolysis as a method to identify ^{32}P-labeled substrates of the tyrosine protein kinases after separation by isoelectric focusing followed by electrophoresis through polyacrylamide gels (Radke and Martin, 1979; Cooper and Hunter, 1981; Cooper et al., 1983). This technique, however, is limited to the study of those proteins that can be focused during isoelectrophoresis. Many glycoproteins do not focus under these conditions, and cell-surface glycoproteins are potentially among the most abundant substrates of the tyrosine protein kinases since most of these kinases reside on the surface of cells. Other studies have used specific antisera to investigate whether or not certain proteins contain phosphotyrosine (Brugge et al., 1981; Sefton et al., 1981; Pasquale et al., 1986). Although this approach is sensitive, it is a very time-consuming method of determining the identity of all proteins that are substrates of tyrosine protein kinases.

Early studies by Ross et al. (1981) showed that antibodies could be elicited to the phosphotyro-

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sine moiety itself. Indeed, more recently, polyclonal antibodies to different phosphotyrosine conjugates have shown that such antibodies could be useful in detecting the substrates of tyrosine protein kinases encoded by oncogenes (Comoglio et al., 1984; Kamps and Sefton, 1988) and the genes for growth factor receptors (Frackelton et al., 1984; White, 1985; Zipple, 1986). Polyclonal antibodies to phosphotyrosine, however, are limited by the fact that all of the antibodies in the mixture must compete for a single antigenic determinant, leaving the lower affinity antibodies in the mixture to give rise to non-specific reactivity. Clearly, monoclonal antibodies to phosphotyrosine offer the possibility of a homogeneous reagent to analyze these modifications. At least three monoclonal antibodies have been reported to phosphotyrosine (Ross et al., 1981; Frackelton et al., 1983; Klarlund, 1985). A detailed characterization of these and other anti-phosphotyrosine-secreting hybridomas has not been reported. In the present study we describe the production of a series of monoclonal antibodies directed to phosphotyrosine that can be used efficiently in both immunoblotting and immunoaffinity purification techniques. These include antibodies of three different subtypes and exhibiting slightly different specificities. These antibodies will be useful in the further identification and analysis of tyrosine phosphorylated proteins.

Materials and methods

Immunizations and fusion protocol

Phosphotyrosine (PY) was coupled to keyhole limpet hemocyanin (KLH), serum albumin (BSA) and ovalbumin (OVA) using EDAC as described (Kamps and Sefton, 1988). The antigen injected into mice consisted of KLH to which equivalent molar amounts of phosphotyrosine, alanine, and glycine had been coupled (Kamps and Sefton, 1988). Using radioactive phosphotyrosine, it was determined that 45 molecules of phosphotyrosine were coupled to each 100 kDa segment of KLH. 200 μg of this antigen was emulsified in 500 μl of 50% complete Freund's adjuvant and used for the first immunization of female BALB/c mice. 1 month later mice were injected with 100 μg of the same conjugate, emulsified in incomplete Freund's adjuvant. After an additional 1 month, 200 μg aliquots of PY-BSA in PBS were injected intravenously each day for 5 days, and 2 days after the last injection the spleen was removed and the cells were fused with the non-secreting cell line, PAI (Stocker et al., 1982). The fusion was performed as described previously (Glenney and Zokas, 1988), using a ratio of 2–3 spleen cells per myeloma cell. After the cells were fused, they were dispersed into 96-well tissue-culture plates, using media containing HAT. 20% of the total cells were plated into ten plates and 80% into another ten plates. Plates were screened for growth, and it was found that the lower dilution contained an average of 2–3 growing hybridomas per well. These ten plates were then screened 8 days after the fusion by ELISA.

Antibody screening

The ELISA was performed by coating Immulon II test plates with 100 ng PY-OVA/well (dried overnight at 37 °C). Culture supernatants were incubated in plates for 1–2 h followed by the addition of HRP-conjugated second antibody for 1 h. The rest of the assay was performed as described previously (Glenney and Zokas, 1988). Color formation in the assay was quantitated with a Dynatech ELISA reader. The 100 hybridomas that elicited the largest colorometric values in the ELISA assay were transferred to 24-well tissue culture plates. Using a Western blotting procedure, antibodies secreted by these clones were next tested for their ability to bind phosphotyrosine-containing proteins derived from NIH3T3 cells, transformed by the tyrosine protein kinase, $p120^{gag\text{-}abl}$. The Western blotting method was essentially as described previously (Glenney and Zokas, 1988), but using 3% BSA, 1% OVA as the blocking agent. The eight hybridomas producing antibodies that reacted most strongly with phosphotyrosine-containing proteins on immunoblots were then cloned by limiting dilution and grown as ascites in mice primed with Freund's adjuvant (Mueller et al., 1986).

Antibody purification

Antibodies were initially purified by three methods. In the first, ascites was applied to a

protein A column. The antibodies that bound to protein A were eluted with a low pH buffer, monitored by absorption at 280 nm, and collected into a tube containing 1.0 M Tris, pH 9.0 (reagents supplies in Affigel protein A Mabs II kit, Bio-Rad). In the second method of purification, IgG was precipitated by the addition of ammonium sulfate to 50%, fractionated on a Sepharose 4B column (monitored by the ELISA assay) and further purified by ion exchange chromatography on a FPLC mono Q column, using a linear gradient of 25–150 mM NaCl in 10 mM Tris, pH 8.0. For the third purification method, ascites fluid was applied to a column of Affigel 10 (Bio-Rad) to which phosphotyramine was coupled. After washing with a large volume of PBS, antibody was eluted by the addition of 1.0 mM phosphotyrosine, pH 7.4. All three methods resulted in an identical pattern of two IgG bands (H & L chain) on SDS-PAGE. After initial testing (Fig. 2), antibodies purified by methods 2 or 3 were used.

Competition ELISA assays

Antibodies were diluted to 50 ng/ml in Tris-buffered saline and preincubated for 1.0 h at 37 °C in the presence of the indicated concentrations of phosphotyrosine, phenylphosphate, phosphoserine or phosphothreonine. A concentration of 50 ng/ml was chosen because at this concentration, half-maximal binding of the anti-phosphotyrosine antibodies to antigen on the ELISA test plates was observed. The solutions were then utilized in an ELISA assay as described above.

^{32}Pi-labeling of cells and immunoprecipitations

5×10^7 SR3T3 cells (BALB/c 3T3 cells transformed by the Schmitt-Rupin strain of RSV, subgroup D) were grown overnight in DMEM lacking phosphate and supplemented with 10% fetal bovine serum and 3.0 mCi ^{32}Pi. After this incubation, the cells were lysed in 8.0 ml of Ripa buffer containing 200 μM sodium *o*-vanadate, and the lysate was clarified by centrifugation at $100\,000 \times g$ for 1.0 h. A portion of this lysate was examined for the abundance of phosphotyrosine in cellular protein by partial acid hydrolysis followed by the separation of phosphoserine, phosphothreonine, and phosphotyrosine by electrophoresis in two-dimensions (Hunter and Sefton, 1980). The lysate was divided into seven portions and each of the seven anti-phosphotyrosine monoclonals were added in the form of 100 μl of a Sepharose : antibody conjugate of 4.0 mg/ml. Reaction between phosphotyrosine-containing proteins and the antibody proceeded for 2 h. The Sepharose was collected by centrifugation and a portion of each of the supernatants was analyzed for the abundance of phosphotyrosine in cellular proteins. The difference between this value and the initial value of total cellular phosphotyrosine was determined to be the percent of phosphotyrosine-containing proteins bound by the respective antibody. Proteins that were bound to the anti-phosphotyrosine : Sepharose matrix were washed four times with 150 mM NaCl; 50 mM Tris, pH 6.8; 0.01% sodium azide; 0.5% Nonidet P-40, 0.5 mM $MgCl_2$ (wash buffer). Phosphotyrosine-containing proteins were then isolated from the resin by two 5 min elutions with 200 μl of wash buffer that contained 20 mM phenylphosphate and 50 μM sodium *o*-vanadate.

Results

In order to generate antibodies to phosphotyrosine (PY) and minimize the number of antibodies generated to carrier proteins, we used a strategy of injecting mice with PY-KLH, boosting with PY-BSA, and screening with PY-OVA. After fusion, the cells were dispersed into ten plates at a low cell concentration (20% of total) and ten plates at a high cell concentration (80%). The plates with the low concentration of cells contained an average of 2–3 growing hybridomas/well, and these were then tested by ELISA, using plates coated with PY-OVA. Approximately 800 of the 1000 wells tested gave rise to reactivity significantly above control levels. 100 of these were expanded and further tested by immunoblotting, using extracts from ANN-1 cells. ANN-1 cells are NIH3T3 cells transformed by a deleted version of the transforming protein encoded by the v-*abl* gene of Abelson murine leukemia virus. The deleted version of this enzyme in ANN-1 cells is a 120 kDa tyrosine protein kinase and is designated p120$^{gag\text{-}abl}$ (Scheer and Siegler, 1975). p120$^{gag\text{-}abl}$ is phosphorylated on tyrosine in vivo

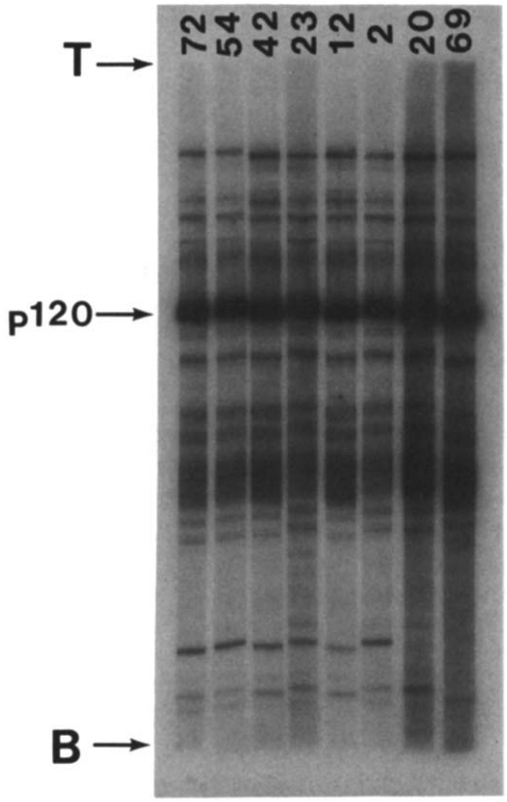

Fig. 1. Western blot analysis using eight different anti-phosphotyrosine antibodies on total cell lysates of ANN-1 cells. Proteins were resolved by SDS-PAGE, transferred to immobilon, blocked with 3% BSA, and reacted with the antibodies (2 μg/ml) listed above each lane. Reactivity was visualized with ^{125}I-labeled anti-mouse antibodies followed by autoradiography. Arrows indicate the top (T) and bottom (B) of the gel as well as the position of the transforming protein, p120$^{gag\text{-}abl}$.

and provides a marker with which immunoreactivity indicates an anti-phosphotyrosine-like activity. These cells exhibit a ten-fold increase in the abundance of phosphotyrosine in total cellular proteins and consequently provide a large number of additional phosphotyrosine-containing proteins that can be recognized by such antibodies. Antibodies displaying the highest reactivity in the immunoblot assay were then cloned and characterized in some detail. As shown in Fig. 1, all of the purified antibodies detected proteins of similar size in lysates prepared from ANN-1 cells.

We then compared three methods of antibody purification for one of the antibodies (py20). As shown in Fig. 2 binding to protein A and elution with low pH resulted in the loss of immunoreactivity when compared to the same antibody purified by either affinity chromatography or classical biochemical methods. No significant difference was observed between affinity-purified antibodies and antibodies that were purified by ammonium sulfate fractionation followed by gel filtration and ion exchange chromatography. We have used the antibody preparations purified by these two methods interchangeably. For convenience, the other seven antibodies were purified by affinity chromatography and their properties were compared.

Subtyping of the antibodies was performed using an ELISA assay and peroxidase-conjugated, subtype-specific second antibodies. Antibody py69 was found to be an IgG2a, py20 an IgG2b, and the rest were of the IgG1 subclass. Since they may represent co-isolates of the same antibody, we further analyzed them by PAGE, under denaturing (SDS) and non-denaturing conditions (Fig. 3). Both heavy and light chains of IgG were observed for all antibodies under denaturing conditions, and for six of the antibodies a single band was observed in non-denaturing buffers. Under non-denaturing conditions, some heterogeneity was observed for two of the antibodies (py69, py23). The

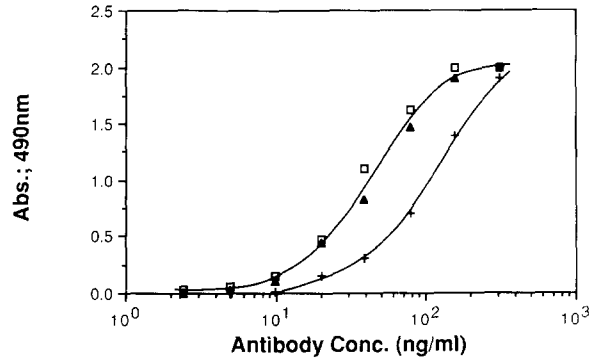

Fig. 2. Comparison of the reactivity of the py20 antibody purified by three different methods. py20 was isolated by (1) salt fractionation, gel filtration and ion exchange chromatography (□), (2) affinity chromatography on a column of affigel-phosphotyramine (▲), or (3) binding to protein A followed by elution with a low pH buffer (+). Antibodies at the specific concentration were then incubated in a 96-well Immulon ELISA plate that was coated with phosphotyrosine, conjugated to ovalbumin. Reactivity was then determined by the standard ELISA assay using peroxidase-conjugated second antibody and *o*-phenylenediamine.

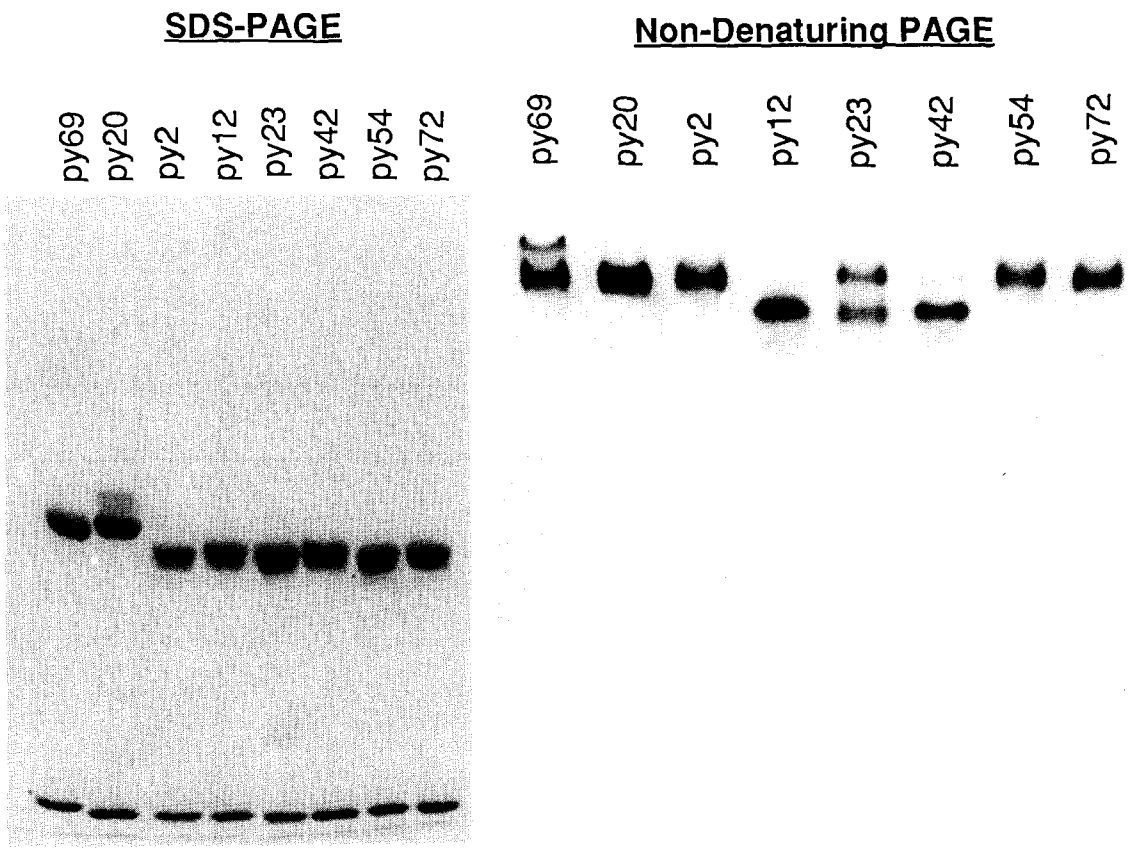

Fig. 3. Electrophoresis of anti-phosphotyrosine antibodies under denaturing or non-denaturing conditions. 5 μg of the antibodies listed above each lane were subjected to electrophoresis in the presence (left) or absence (right) of SDS. Protein was visualized with Coomassie blue dye.

reason for this heterogeneity is unknown. Of the IgG1 antibodies tested, at least three could be distinguished by their electrophoretic mobility. From this type of analysis we could not tell whether those that have a similar electrophoretic pattern represent the same antibodies.

To determine the specificity of the antibodies for different phosphoamino acids, a competition ELISA assay was performed. Antibodies were preincubated with phosphoserine, phosphothreonine, or phosphotyrosine, and then the mixture was added to a test plate containing PY-OVA. As shown in Fig. 4 all of the antibodies tested could be inhibited by preincubation with phosphotyrosine and phenylphosphate, but not by phosphoserine or phosphothreonine. In all cases, phosphotyrosine was a slightly more potent inhibitor then phenylphosphate. Most of the antibodies displayed a similar sensitivity toward phosphotyrosine with half maximal inhibition occurring between 50–200 μM phosphotyrosine. In these assays the antibody concentration was 0.3 nM, thus in all assays the inhibitor was present at a large molar excess.

The antibodies were also useful for identifying and isolating phosphoproteins from cells. To assess the specificity of binding of phosphoproteins to these antibodies, they were coupled to Sepharose 4B at a concentration of 4.0 mg/ml of Sepharose and used to isolate phosphotyrosine-containing proteins from SR3T3 cells, labeled with ^{32}Pi. As shown in Fig. 5, phosphorylated proteins bound to the affinity resin and could be eluted with phenylphosphate. Only minor differences were noted in the profile of phosphoproteins that bound to the different antibodies, yet this profile was markedly

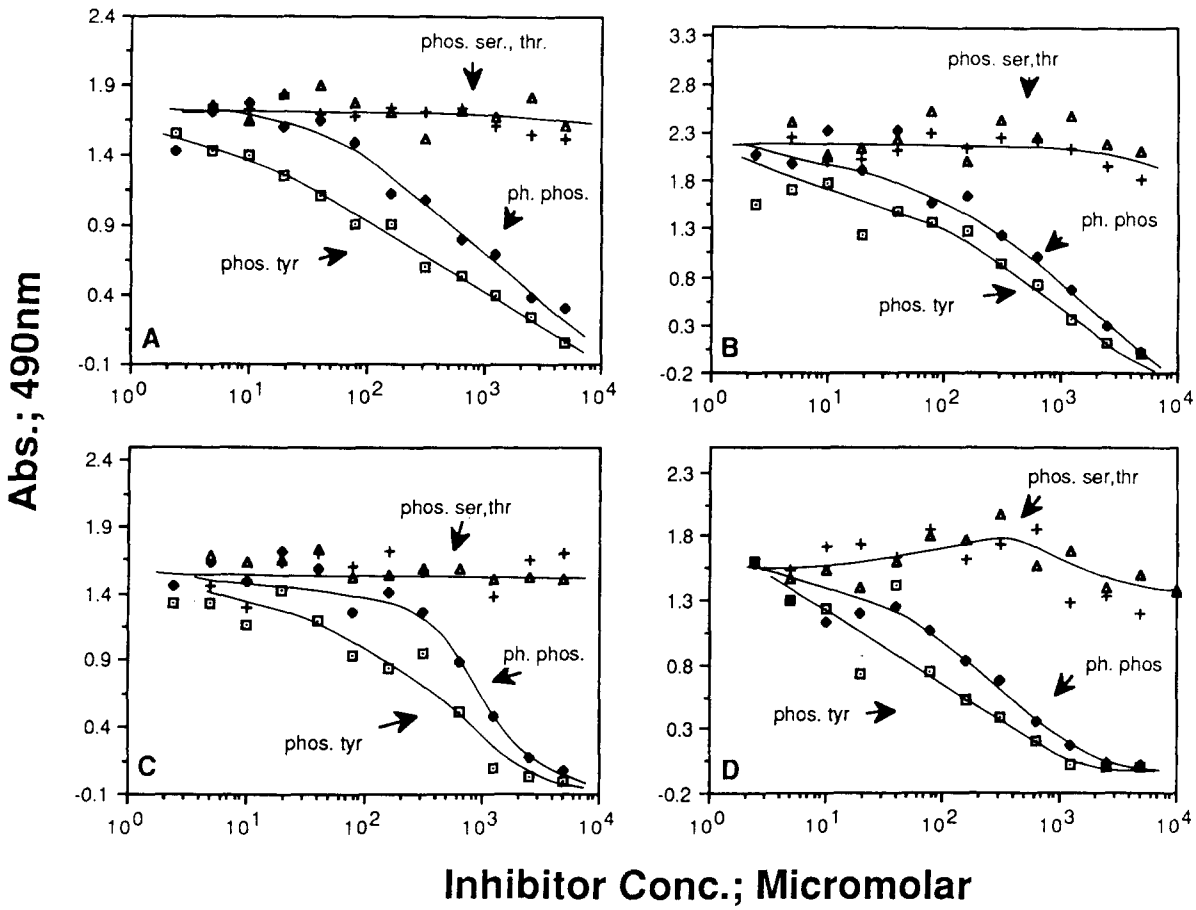

Fig. 4. Inhibition of antibody binding by phosphoamino acids. The antibodies py69 (*A*), py20 (*B*), py2 (*C*), or py12 (*D*) at 50 ng/ml (0.3 nM) were preincubated with the specified concentration of phosphotyrosine (□), phosphoserine (△), phosphothreonine (+), or phenylphosphate (◆), and the solution was then added to the ELISA assay as described in the legend to Fig. 2. All data points represent the sum of duplicate determinations.

different from the starting material (lane 1). Phosphoamino acid analysis demonstrated that phosphotyrosine was depleted in the unbound protein and comprised approximately 25% of the acid-stable phosphoamino acids in the phosphoproteins that were eluted from the columns by competition with phenyl phosphate (Table I).

The observation that 75% of the phosphoamino acids were phosphoserine and phosphothreonine can be attributed either to the fact that phosphotyrosine-containing proteins are also phosphorylated on serine and threonine, or to the possibility that other cellular phosphoproteins bind to the antibodies in a non-specific fashion. We addressed these two alternatives by isolating phosphotyrosine-containing proteins from SR-3T3 cells grown in the presence or absence of sodium *o*-vanadate. Sodium *o*-vanadate is a phosphatase inhibitor that is more inhibitory to phosphotyrosine phosphatases than to phosphoserine/phosphothreonine phosphatases (Leis and Kaplan, 1982; Swarup et al., 1982). When SR3T3 cells are grown in the presence of sodium *o*-vanadate the percentage of phosphotyrosine in protein increases approximately eight-fold relative to phosphoserine and phosphothreonine. We found that four times as much total phosphoprotein was isolated from cells grown in the presence of vanadate (data not shown). If the phosphoserine and phosphothreonine that we observe in proteins that are eluted

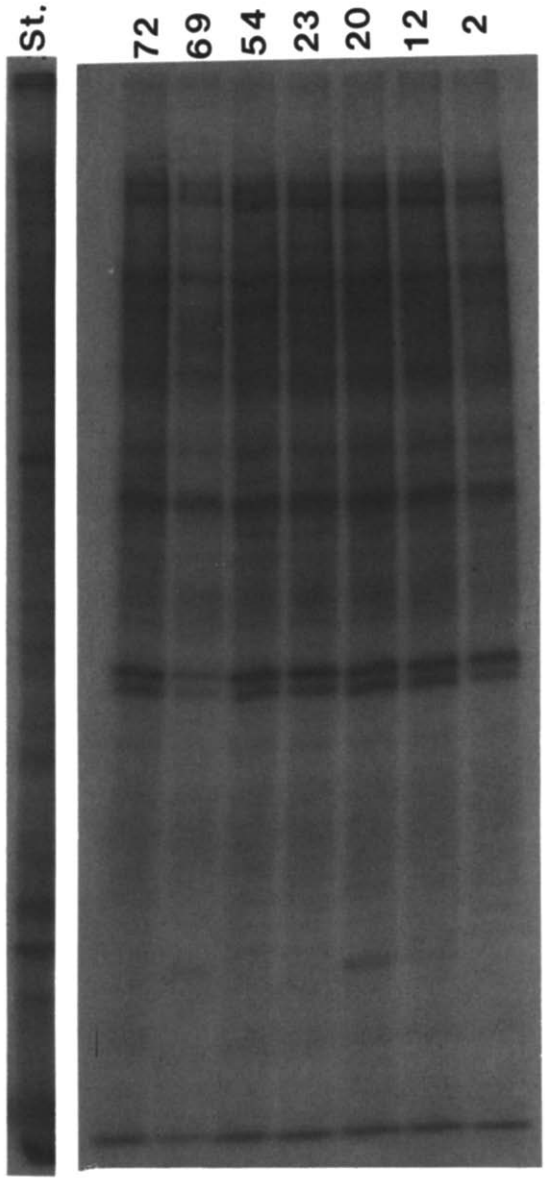

Fig. 5. Purification of phosphotyrosine-containing proteins using anti-phosphotyrosine antibodies coupled to Sepharose and extracts from SR3T3 cells, grown in the presence of ^{32}Pi. The lane designated 'St' represents the pattern of phosphoproteins obtained from 5×10^3 cells of extract. The lanes with numeric designation exhibit phosphotyrosine-containing proteins purified from 5×10^5 cells. Each of the numbers represents the identity of the monoclonal antibody that was used.

from antiphosphotyrosine antibodies is contained in the same proteins that are phosphorylated on tyrosine, then the percentage of phosphoserine

TABLE I

QUANTITATION OF PHOSPHOTYROSINE IN CELLULAR PROTEINS BEFORE AND AFTER ADSORPTION TO ANTIPHOSPHOTYROSINE ANTIBODIES

The column designated 'unbound' represents the percent phosphotyrosine in cellular proteins before (control) and after (numeric antibody designations) adsorption to the anti-phosphotyrosine: Sepharose resins. The column designated 'eluted' represents the percentage phosphotyrosine in the proteins removed from the phosphotyrosine: Sepharose resins by competition with phenylphosphate.

Antibody	% Phosphotyrosine	
	Unbound	Eluted
Control	1.1	–
py69	0.73	29
py20	0.62	24
py2	0.67	25
py12	0.58	23
py23	0.63	23
py54	0.70	24
py72	0.63	24

and phosphothreonine in proteins that bind to the antibody from cells treated with vanadate should be identical to those observed in proteins derived from cells grown in the absence of vanadate. However, if the phosphoserine and phosphothreonine is derived from proteins that bind to the antibody non-specifically, the relative abundance of phosphotyrosine in proteins isolated from cells treated with vanadate should be substantially enriched. Although four times as much total phosphoprotein was isolated with antiphosphotyrosine antibodies from cells treated with vanadate, the relative abundance of phosphotyrosine was the same, 30% in both cases (data not shown). Consequently, these antibodies are specific for phosphotyrosine, and proteins phosphorylated on tyrosine also contain substantial amounts of phosphoserine and phosphothreonine.

Discussion

In order to analyze the substrates of oncogenic and growth factor receptor tyrosine kinases we have generated a series of monoclonal antibodies to phosphotyrosine. We report here the pre-

liminary characterization of these antibodies. Of the 800 hybridomas that were initially observed to secrete antibodies to phosphotyrosine, we selected eight (1%) that displayed the strongest reactivity by both ELISA and Western blotting assays. These were then grown as ascites in mice and the properties of purified antibodies were tested by Western blotting, hapten competition assays, and immunoaffinity chromatography.

Each of the eight selected antibodies displayed a similar specificity for phosphorylated proteins and a similar affinity for phosphotyrosine alone. The most appropriate antibody to use in subsequent investigations, then, will be dictated by convenience. IgG1 antibodies, for instance, do not bind strongly to protein A, while IgG2a and IgG2b do. The antibodies py69 and py20 then may be useful when a protein A detection system is to be employed.

Differences in reactivity of the various antibodies with minor immunoreactive components displayed on Western blots were observed. This difference could reflect effects of the surrounding amino acids which prevent some of the antibodies from binding. This would then be a consideration in using the antibodies to detect unknown phosphoproteins by Western blotting. For this purpose, monoclonals py2, py23, and py54 appeared to be the best.

These antibodies should find widespread use in analyzing various aspects of oncogenesis and growth factor mediated signal transduction. Two of the antibodies (py69, py20), for instance, have been shown to block EGF-mediate endocytosis when microinjected into cells (Glenney et al., 1988). The antibodies were further shown to bind directly to the activated EGF receptor and prevent subsequent substrate phosphorylation in vitro. This study also demonstrated that cells treated with EGF contained numerous proteins that bound to py20 on Western blots whereas untreated cells contained only a few nonabundant proteins that were reactive with the antibody. This result affirmed the specificity of the antibodies towards proteins containing phosphotyrosine. In addition, we have found that the purified receptor for EGF, when phosphorylated on tyrosine in vitro, will bind to a column of py20-Sepharose quantitatively, and can be eluted with phosphotyrosine. We are currently using these antibodies to purify novel substrates of the tyrosine kinases for the production of second generation monoclonals.

Acknowledgements

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