MOLECULAR AND CELLULAR BIOLOGY, Dec. 1985, p. 3640–3643
0270-7306/85/123640-04$02.00/0

Vol. 5, No. 12

Isolation of Antibodies for Phosphotyrosine by Immunization with a v-*abl* Oncogene-Encoded Protein

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Received 21 June 1985/Accepted 29 August 1985

Immunization of rabbits with a tyrosine-phosphorylated v-*abl* protein resulted in the production of antibodies for the v-*abl* protein and for phosphotyrosine. The antiphosphotyrosine antibodies could be purified by affinity chromatography with *O*-phosphotyramine coupled to Sepharose. These antibodies detected a variety of tyrosine-phosphorylated proteins, including receptors for peptide growth factors. The usefulness of these antibodies was demonstrated by the detection of previously unidentified tyrosine-phosphorylated proteins in v-*src*-, v-*abl*-, and v-*erbB*-transformed cell lines.

Tyrosine phosphorylation of cellular proteins is a special form of covalent modification, which appears to play an important role in the regulation of cell growth. Cell surface receptors for several different peptide growth factors are tyrosine kinases whose activities are regulated by their ligands (12). Other tyrosine kinases, e.g., the proteins encoded by the v-*src* or v-*abl* oncogenes, can induce the transformation of normal fibroblasts or lymphoid cells (1). Although the importance of these tyrosine kinases in growth regulation has been demonstrated, the mechanism of their action is not understood. A useful probe for the study of protein tyrosine phosphorylation is an antibody which recognizes phosphorylated tyrosine residues in proteins. Several groups of investigators have generated antibodies to *p*-azobenzyl phosphonate (ABP), an analog of phosphotyrosine (PTyr), and have shown that these antibodies can react with specific tyrosine-phosphorylated proteins (2, 7, 8, 19). Here I describe the isolation of anti-PTyr antibodies which were produced by rabbits immunized with a v-*abl* oncogene-encoded protein.

The v-*abl* oncogene of Abelson murine leukemia virus (A-MuLV) is a member of the tyrosine kinase gene family (1, 18, 22). The v-*abl* coding sequence has been expressed in bacteria from which a large quantity of a tyrosine-phosphorylated v-*abl* protein can be generated (22). Immunization of female New Zealand White rabbits with this tyrosine-phosphorylated v-*abl* protein was carried out by injecting 200 μg of antigen into the lymph nodes, followed by a boost of 2 mg of antigen 4 weeks later. This immunization program resulted in the production of anti-v-*abl* antibodies. Immune sera from the rabbits reacted with v-*abl*-encoded proteins produced by A-MuLV or produced in bacteria. Reactions between the v-*abl* proteins and the antisera could be blocked by the addition of excess antigen. Although the immunogen was a tyrosine-phosphorylated v-*abl* protein, the antisera recognized non-tyrosine-phosphorylated forms of the v-*abl* proteins as well (data not shown).

The presence of anti-PTyr antibodies in these immune sera was indicated by the results shown in Fig. 1. In this experiment, BALB/c 3T3 cells were stimulated with platelet-derived growth factor (PDGF) for 10 min at 37°C. They were then harvested at the same time as a parallel unstimulated culture and lysed immediately in boiling sodium dodecyl sulfate (SDS) buffer containing 2% SDS, 0.3% 2-mercaptoethanol, 10 mM EDTA, and 66 mM Tris hydrochloride at pH 6.8. These lysates were subjected to SDS-polyacrylamide gel electrophoresis, and the protein bands were transferred onto nitrocellulose filters (21). The nitrocellulose filters were incubated with either the antiserum or the corresponding preimmune serum. The bound antibody was then probed with ^{125}I-labeled goat anti-rabbit antibody. The antiserum, but not the preimmune serum, reacted with a 180-kilodalton (kDa) protein in lysates of PDGF-stimulated cells. However, this protein was not recognized by the antiserum in unstimulated cells (Fig. 1). PDGF is known to stimulate tyrosine phosphorylation of its receptor, a 180-kDa membrane protein (6, 16, 25). Since the immune serum only recognized the stimulated 180-kDa protein and the only known difference between the stimulated and unstimulated PDGF receptor is tyrosine phosphorylation, we postulated that there were anti-PTyr antibodies in the immune serum.

If the interaction of the 180-kDa protein with the immune serum was through a PTyr residue in the protein, the immune reaction should be blocked by PTyr or its analogs, e.g., phenylphosphate. In both immunoprecipitation and immunoblotting experiments, 10 mM PTyr or phenylphosphate completely inhibited the binding of antibodies to the 180-kDa protein. Phosphoserine and phosphothreonine at comparable or much higher concentrations did not have any effect. The antiserum also reacted with epidermal growth factor (EGF) receptor and insulin receptor after they were stimulated with their respective ligands (data not shown). These immune reactions were inhibited by PTyr as well. Immunoprecipitations of A-MuLV-encoded proteins, on the other hand, were only partially inhibited by PTyr or phenylphosphate. This would be expected if the immune serum contained both anti-v-*abl* and anti-PTyr antibodies.

The anti-PTyr antibody in the immune sera was purified by using the phosphotyramine-Sepharose affinity column as described by Ross et al. (19). Approximately 500 μg of antibody was bound to the column and could be eluted with 40 mM phenylphosphate when 25 ml of antiserum was processed. The yield of anti-PTyr antibody varied from 50 to 500 μg/25 ml of serum, depending on the rabbit and the time of bleeding. The affinity-purified antibody from different rabbits reacted with tyrosine-phosphorylated proteins with the same specificity and the same apparent affinity. In the flowthrough fraction of the column, anti-v-*abl* antibody activity was detected. This was shown by the immunoprecipitation of a 160-kDa *gag*-v-*abl* fusion protein in an A-MuLV-transformed cell line, N54 (14). The 160-kDa viral protein could be labeled with ^{32}P by incubating N54 cells

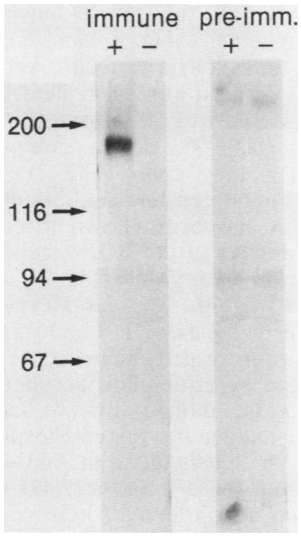

FIG. 1. Antiserum against the v-*abl* protein reacted with stimulated receptor for PDGF. BALB/c 3T3 cells were stimulated with PDGF as previously described (8). Lysates of stimulated (+) or unstimulated (−) cells were immunoblotted with a 1:500 dilution of the immune serum or preimmune serum, as indicated in the figure. Protein molecular mass standards are for myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase *a* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and carbonic anhydrase (30 kDa).

with $^{32}P_i$ in phosphate-free medium for 1 h at 37°C. The labeled cell lysates were prepared, and the proteins were immunoprecipitated as described previously (23). The 160-kDa *gag*-v-*abl* protein was recognized by both the flowthrough and the bound fractions of the phosphotyramine-Sepharose column (Figure 2I, lanes B and C). Antibody from the bound fraction was completely inhibited by phenylphosphate (lane c), whereas reaction with the antibody from the flowthrough fraction was not affected by phenylphosphate (not shown) but was inhibited by the v-*abl* antigen (lane b). Separation of these two populations of antibodies demonstrated that only the anti-PTyr antibody reacted with stimulated PDGF receptor (Fig. 2III). Although the flowthrough fraction immunoprecipitated the A-MuLV protein, it did not recognize the PDGF-stimulated 180-kDa protein (lane B).

To examine the PTyr specificity, I used the affinity-purified antibodies to immunoprecipitate ^{32}P-labeled proteins from v-*src*-transformed cells (SR3T3 line). A number of labeled proteins ranging from 210 to 32 kDa reacted with the antibody, and their immunoprecipitation could be blocked by phenylphosphate (Fig. 2II, lanes C,c). The radiolabel in these bands was partially resistant to alkaline treatment (1 N KOH at 55°C for 2 h), indicating that they contained PTyr in addition to other phosphoamino acids. Phosphoamino acid analysis of the immunoprecipitated proteins confirmed this conclusion. Proteins brought down by the anti-PTyr antibody had an average phosphoserine-to-phosphothreonine-to-PTyr ratio of 1.8:0.4:1. This ratio was comparable to that of immunoprecipitated pp60^{src} (1.5:0.3:1) or immunoprecipitated pp36 (2.1:0.2:1) determined in parallel experiments with the same ^{32}P-labeled SR3T3 cell lysates and antisera specific for these two proteins. Proteins immunoprecipitated with an irrelevant antiserum had a ratio of 20:5:1. Although pp60^{src} and pp36 have been shown to be tyrosine phosphorylated in v-*src*-transformed cells (3), they were not immunoprecipitated by the anti-PTyr antibody described here. None of the immunoprecipitated bands comigrated with pp60^{src} (Fig. 2II, lanes T and C) or pp36 (data not shown). It appears that these anti-PTyr antibodies only recognize a subset of tyrosine-phosphorylated proteins. Since they were generated against a tyrosine-phosphorylated v-*abl* protein, the binding sites of these antibodies might be designed for an epitope larger than PTyr. The structure surrounding the PTyr in proteins such as pp60^{src} or pp36 might not be compatible with the binding sites of these anti-PTyr antibodies, whereas other tyrosine-phosphory-

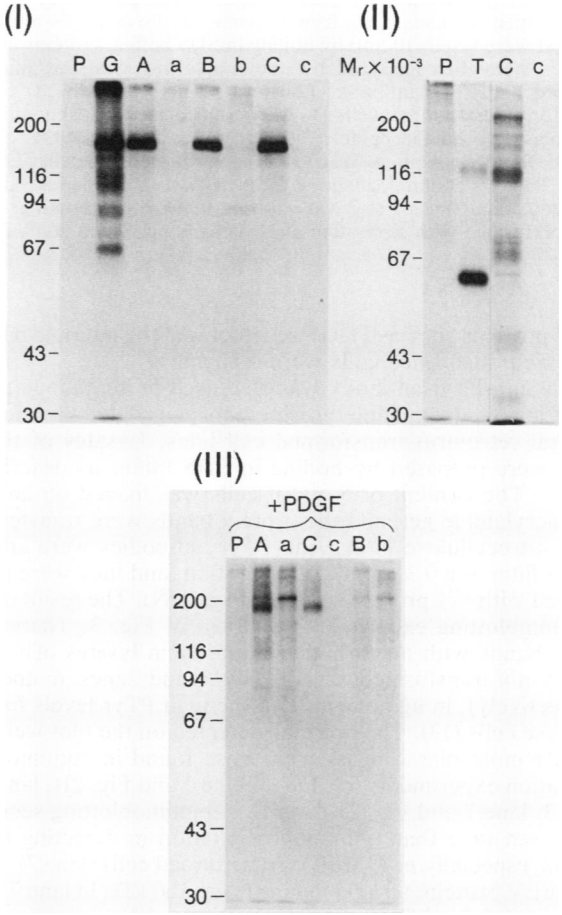

FIG. 2. Presence of anti-v-*abl* and anti-PTyr antibodies in the immune sera. Antibodies from the immune sera were fractionated on a phosphotyramine-Sepharose column as previously described (19). The unbound fraction and the bound fraction of the column were used to immunoprecipitate lysates of three different cells pulse-labeled with $^{32}P_i$ for 1 h at 37°C. (I) A-MuLV(P160)-transformed fibroblasts, line N54. (II) RSV-transformed mouse fibroblasts, line SR3T3. (III) PDGF-stimulated NIH 3T3 cells. Lanes: P, preimmune serum; A, immune serum; a, immune serum plus an excess of v-*abl* antigen; B, unbound fraction of the phosphotyramine-Sepharose column; b, unbound fraction plus an excess v-*abl* antigen; C, antibody affinity-purified from the phosphotyramine-Sepharose column; c, affinity-purified antibody plus 40 mM phenylphosphate; G, goat anti-Moloney murine virus serum which recognizes the *gag*-v-*abl* fusion protein of A-MuLV through its *gag* determinants (26); T, tumor-bearing rabbit serum which contained antibody for pp60^{src}.

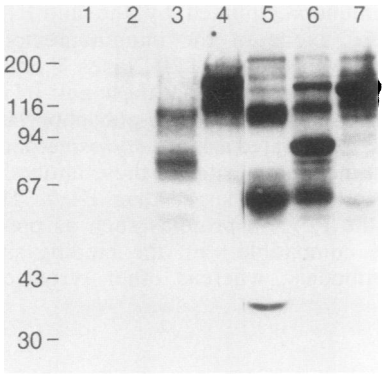

FIG. 3. Detection of tyrosine-phosphorylated proteins in several transformed cell lines. Cells from a sparse culture plated 24 h before harvest were lysed (10^7/ml) by boiling in SDS buffer. Proteins in the lysates were then analyzed by immunoblotting with the affinity-purified anti-PTyr antibody. Lanes: 1, NIH 3T3 cells; 2, SSV-transformed rat kidney cells; 3, AEV-transformed chicken embryo fibroblasts; 4, human epidermoid carcinoma cell line A431, clone 29R; 5, SR3T3 cells; 6, A-MuLV(P90)-transformed 3T3 cells; 7, N54 cells. The blot containing lanes 1 through 4 was exposed for 3 days; that containing lanes 5 to 7 was exposed for 18 h. Autoradiography was performed with X-ray film plus intensifying screen at −80°C.

lated proteins such as PDGF receptor and the multiple bands in v-*src*-transformed cells were recognized.

The anti-PTyr antibody was also used in immunoblotting experiments to examine tyrosine-phosphorylated proteins in several retrovirus-transformed cell lines. Lysates of these cells were prepared by boiling in SDS buffer as described above. The content of 5×10^5 cells was loaded on an 8% polyacrylamide gel, and the protein bands were transferred onto nitrocellulose filter. Anti-PTyr antibodies were added to the filter at a 0.3 μg/ml concentration, and they were later probed with ^{125}I-protein A (10 μCi/μg; ICN). The result of an immunoblotting experiment is shown in Fig. 3. There are more bands with much higher intensity in lysates of v-*src*- and v-*abl*-transformed cells (lane 5 and lanes 6 and 7, respectively), in agreement with the high PTyr levels found in these cells (13). The proteins detected on the blot were of similar molecular weights with those found in immunoprecipitation experiments (cf. Fig. 3, lane 5 and Fig. 2II, lane C; Fig. 3, lane 7 and Fig. 2I, lane C.). Immunoblotting seemed more sensitive than immunoprecipitation in detecting faint bands, especially in A-MuLV-transformed cells (lane 7). The A-MuLV protein, 90 kDa in lane 6 and 160 kDa in lane 7 (for a review of the different variants of A-MuLV, see reference 11), was the most prominent tyrosine-phosphorylated protein in v-*abl*-transformed cells. In v-*src*-transformed cells, these antibodies reacted well with two bands with molecular masses of ca. 116 kDa and two of ca. 65 kDa. The prominent bands at 65 kDa were not pp60^{src}. I could show, in control experiments, that pp60^{src} was not detected by the antibody when it was isolated and immobilized onto nitrocellulose filters. The 46-kDa proteins found in the immunoprecipitates (Fig. 2II, lane C) could also be found on the blot of a longer exposure. The higher intensity of these 46-kDa bands in a pulse-labeled cell lysate might indicate that the tyrosine phosphates in these proteins had a faster turnover rate, thus the PTyr content of these proteins was lower at steady state. Several protein bands were common to both v-*src*- and v-*abl*-transformed cells. These include 210-, 170-, and 32-kDa bands. The 32-kDa band was much more prominent in v-*src*-transformed cells (Fig. 3, lanes 5 to 7).

After an eight-times-longer exposure, prominent tyrosine-phosphorylated proteins were also detected in lysates of avian erythroblastosis virus (AEV)-transformed chicken embryo fibroblasts (lane 3), simian sarcoma virus (SSV)-transformed rat kidney cells (lane 2), and the human epidermoid carcinoma cell line A431 (lane 4). The v-*erbB* oncogene of AEV has been shown to correspond to the tyrosine kinase domain of the EGF receptor (5), yet AEV-transformed chicken embryo fibroblasts do not contain elevated levels of PTyr (10). The anti-PTyr antibody reacted with two prominent bands of 110 and 72 kDa and several minor bands. Untransformed chicken embryo fibroblasts did not contain these tyrosine-phosphorylated proteins (not shown). Note that the antibody did not react with a 36-kDa band, despite the finding of tyrosine-phosphorylated pp36 in these cells (10). The bands found in AEV-transformed cells were different from those found in A431 cells. This carcinoma cell line has a high level of EGF receptor, due to an amplification of the receptor gene (21). At steady state and without deliberate stimulation with EGF, a protein of 150 kDa, the size of the receptor, and several other minor bands could be detected by the anti-PTyr antibody in A431 cells. The oncogene v-*sis* of SSV encodes a protein which is homologous with one chain of PDGF (4). It has been shown that the v-*sis* protein can interact with PDGF receptor and stimulate tyrosine phosphorylation of the 180-kDa receptor protein (9, 17, 24). The anti-PTyr antibody only reacted with a very faint band of 175 kDa in SSV-transformed cells. It seems that v-*sis*-mediated transformation does not involve major alterations in the tyrosine phosphorylation of cellular proteins. This is in agreement with the finding that the PDGF receptor is down-regulated in SSV-transformed cells (9).

Previous studies by Cooper and Hunter with two-dimensional gels have led to the identification of several tyrosine-phosphorylated proteins in v-*src*- and v-*abl*-transformed cells (3). These include vinculin (130 kDa), lactate dehydrogenase (35 kDa), phosphoglycerate mutase (28 kDa), enolase (46 kDa), and proteins of 81, 50, and 36 kDa. Ross et al. isolated a 116-kDa protein from Rous sarcoma virus (RSV)-transformed 3T3 cells with their anti-ABP antibodies coupled to Sepharose (19). Comoglio et al. have found tyrosine-phosphorylated proteins with molecular masses of 130, 70, and 60 kDa in the detergent-insoluble fraction of RSV-transformed cells by using their anti-ABP antibodies (2). It appears that different methods and even different anti-ABP antibodies detected different tyrosine-phosphorylated proteins. It is conceivable that not all of the tyrosine-phosphorylated proteins in RSV- or A-MuLV-transformed cells have been described. The anti-PTyr antibodies described here detected bands corresponding to the sizes of some of the previously described tyrosine-phosphorylated proteins. In addition, these new anti-PTyr antibodies reacted well with several other proteins, including a 210-kDa band in both v-*src*- and v-*abl*-transformed mouse cells.

Although it is not evident in Fig. 3, these anti-PTyr antibodies can also react with tyrosine-phosphorylated proteins in untransformed cells (A. O. Morla and J. Y. J. Wang, unpublished data). These antibodies have also been used in immunofluorescence studies, where they have yielded the interesting finding that PTyr-containing proteins are present at cell-substratum and cell-cell junctions in normal cells (14a), a result not observed with anti-ABP antibodies prepared by Marchisio et al. (15). Because these anti-PTyr

antibodies provide the sensitivity to detect tyrosine-phosphorylated proteins in normal cells, they should be useful in future studies of the role of tyrosine kinases in the regulation of cell growth.

I am grateful to Lewis T. Williams of the Howard Hughes Medical Institute, University of California, San Francisco, for his helpful discussions throughout this work and his generous gift of PDGF and phosphotyramine-Sepharose and to George Anders for his help in immunizing rabbits.

This work was supported by National Science Foundation grant PCM 83-14300 and a grant from the Camille and Henry Dreyfus Foundation. J.Y.J.W. is a Searle Scholar.

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