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A Monoclonal Antibody Reactive With an Activated Ras Protein Expressing Valine at Position 12

W.P. Carney, P. Hamer, D. Petit, H. Wolfe, G. Cooper, M. Lefebvre, and H. Rabin

Biomedical Products Department, E.I. du Pont de Nemours and Company, Inc., North Billerica, Massachusetts 01862 (W.P.C., P.H., D.P., H.R.), Department of Pathology, Dana Farber Cancer Institute (G.C., M.L.), and Tufts University Medical School (H.W.), Boston, Massachusetts 02115

Activated ras transforming genes have been described in a variety of neoplasms and encode 21,000-Dalton (p21) proteins with amino acid substitutions at positions 12, 13, and 61. In this report we describe a monoclonal antibody designated DWP that reacts specifically with synthetic dodecapeptides containing valine at position 12, to a lesser extent with peptides containing cysteine at position 12 and not with peptides containing glycine, arginine, serine, aspartic acid, glutamic acid or alanine at the same position. Western blot and immunoperoxidase studies showed that DWP specifically reacts with activated ras^H or ras^K proteins in NIH cells transformed by DNA from the human carcinoma cells that encode valine at position 12. DWP did not react with normal p21s encoding glycine at position 12, nor with activated p21s encoding aspartic acid, glutamic acid, arginine, serine, or cysteine at position 12. A survey of human tumor cell lines demonstrated that DWP reacted with the human bladder carcinoma cell line T24 but not with human tumor cell lines previously shown to contain other activating mutations at positions 12 or 61. DWP and perhaps additional antibodies that specifically react with alterations at positions 12 or 61 of the ras protein may be valuable in determining the presence and frequency of activated ras proteins in human malignancy.

Key words: monoclonal antibody DWP, activated ras protein reactive antibody, anti-ras antibodies, anti-ras monoclonal antibody

Ras genes homolgous to the Harvey (Ha) and Kirsten (Ki) sarcoma virus oncogenes [1] have been described in a variety of organisms including yeast [2], fruit flies [3], and mammalian cells [4]. In mammalian cells the ras genes encode 21,000-Dalton proteins that are localized to the plasma membrane [5], bind guanine nucleotides [6–8], and mediate GTPase activity [9–12]. DNA transfer experiments have demonstrated that a wide variety of neoplastic cells, including sarcomas, neuroblas-

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tomas, carcinomas, and hematopoietic malignancies [reviewed in 13], contain structurally altered ras genes, which upon transfection into NIH 3T3 cells lead to transformation. These oncogenic ras genes differ from their normal counterparts by point mutations that result in amino acid alterations at positions 12, 13, or 61 of the protein product [14–17]. Since activated ras gene products differ from their normal homologs, we directed our efforts at developing monoclonal antibodies (MOAb) that could discriminate between activated and normal proteins. In a preliminary report [18] we described a MOAb, DWP, that was raised against a ras-related synthetic peptide corresponding to an activated ras protein containing valine at position 12. In this report we extend our investigations and characterizations of DWP to a variety of transformed NIH cells and human carcinoma cells containing either activated or normal ras proteins. Our results demonstrate that DWP reacts specifically with cells having activated ras proteins containing valine at position 12 and not with normal p21s or other activated p21s.

MATERIALS AND METHODS

Hybridoma Production and Selection

To select antibodies specific for activated p21s containing valine at position 12, Balb/c × C57BL/6 mice were immunized with a ras-related synthetic peptide ^{5}Lys-Leu-Val-Val-Val-Gly-Ala-Val-Gly-Val-Gly-Lys^{16} corresponding to positions 5–16 of the activated ras protein in the T24 bladder carcinoma and shown to contain valine at position 12. Peptides were coupled to the carrier protein bovine thyroglobulin (BTG) for immunization and to keyhole limpet hemocyanin (KLH) for screening assays. This strategy avoids selection of antibodies to the BTG carrier protein. Peptides were coupled to carrier proteins using l-ethyl-3-(3-dimethyl amino propyl) carbodiimide hydrochloride as previously described [19]. The immunization schedule consisted of inoculating mice intraperitoneally (ip) on days 1, 14, 28, 42, and 59 with 100–200 μg of conjugate. Inoculations on days 1 and 14 consisted of the peptide carrier-protein conjugate mixed 1:1 with complete Freunds adjuvant whereas the remaining inoculations consisted of conjugate mixed with phosphate buffered saline (PBS). Mouse sera were collected and evaluated by enzyme-linked immunosorbent assay (ELISA) [20] for the presence of antipeptide antibody. Three days prior to fusion mice were inoculated ip with 100 μg of peptide–BTG conjugate. On the day of fusion, spleens were removed and single cell suspensions of immune spleen cells were fused with Sp2/0 cells using polyethylene glycol as previously described [21].

Two to three weeks after cell hybridizations, hybridoma supernatants were evaluated by ELISA for binding to KLH-coupled peptides (500 ng/well) containing either valine or glycine at position 12. The hybridoma secreting the antibody of interest was doubly cloned by limiting dilution, inoculated into Pristane-primed mice, and the resultant ascites fluid used to prepare purified immunoglobulin [22].

Immunohistochemical Staining

Immunoperoxidase studies employed the avidin biotin complex system as previously described [23]. NIH cells and human tumor cell lines were formalin-fixed and embedded in paraffin prior to evaluation. All studies included a class-matched

myeloma protein MOPC 141 (Litton Bionetics, Rockville, MD) at identical protein concentrations as DWP.

Cell Cultures

All cells were maintained in Dulbecco's modified Eagle's media supplemented with 5% fetal calf serum.

Production of Nude Tumors

Ten million tissue culture grown cells were resuspended in 0.2ml of PBS and inoculated subcutaneously into nude mice. Tumors were removed 2–4 weeks later and processed as described for the Western blot.

Western Blot

Cell extracts from 10^9 cells were prepared by scraping cells into Triton X-100 lysis buffer and the p21s were concentrated from cell extracts by immunoprecipitation with the broadly reactive anti-ras MOAb, Y13-259 [24,25]. For Western blot analysis, immunoprecipitates were collected, washed, and boiled in sample bufffer containing 2-mercaptoethanol. Immunoprecipitated proteins as well as heavy and light immunoglobulin chains were resolved by SDS-PAGE on 12.5% polyacrylamide gels and transferred to nitrocellulose membranes. After blocking with PBS containing 5% bovine serum albumin (BSA) membranes were incubated for 1 hr with 25 μg/ml of Y13-259, DWP, or MOPC 141. Membranes were washed three times with PBS-NP-40 (0.05%), incubated with either rabbit anti-rat horseradish peroxidase (HRP) to detect Y13-259 or goat anti-mouse HRP for 1 hr to detect DWP and MOPC 141. Membranes were then washed three times with PBS-NP-40, and incubated with 4-chloro-1-napthol substrate to complete the reaction as previously described [26].

RESULTS

Balb/c × C57B1/6 mice were immunized with a synthetic dodecapeptide corresponding to positions 5–16 of the activated ras protein described in T24 human bladder carcinoma cells. This protein expresses the valine substitution at position 12. Mice that exhibited serum reactivity with the immunogen of greater than 1:50,000 were selected for hybridoma production. Hybridoma supernatants were tested by ELISA for the presence of antibodies that would selectively bind peptides (coupled to KLH) containing valine at position 12 but not peptides containing glycine at position 12. One hybridoma, designated DWP, secreted an antibody with the desired reactivity pattern and was therefore doubly cloned and used to produce purified immunoglobulin from ascites fluid as previously described [22]. Purified DWP was determined by ELISA to be an IgG2b kappa molecule using rabbit antibodies against various classes of mouse immunoglobulins.

DWP was initially characterized by titration on peptides containing either valine or glycine (normal) at position 12. Results presented in Table I show that as little as 5 pg of purifed DWP reacted by ELISA with unconjugated peptide containing valine at position 12, whereas DWP did not react with glycine-position 12 peptides even when as much as 50 μg of peptides was used (data not shown).

To define DWP specificity further, a competition assay was performed using peptides containing a variety of amino acid substitutions at position 12. To do this,

TABLE I. Titration of DWP on RAS-Related Peptides Containing Either Valine or Glycine at Position 12

DWP concentration (ng/ml)	Optical density (488 nm)	
	Peptide-Val-12	Peptide-Gly-12
50	1.8	0
5	1.5	0
0.5	1.0	0
0.05	0.8	0
0.005	0.4	0

Various concentrations of purified DWP were incubated with 100 ng of unconjugated peptides coated onto the surface of microtiter wells. After an overnight incubation at 4°C, plates were washed and the goat anti-mouse horseradish peroxidase conjugate added for 1 hr. Plates were then washed and the substrate o-phenylene-diamine was added to complete the reaction. Colorimetric changes were determined using an ARTEK plate reader at 488 nm.

1.75μg of DWP was mixed wtih various peptides (concentrations ranging from 15.6 -500 ng) and allowed to incubate for 2 hr at 37°C. After the incubation, fluids containing peptides and DWP were incubated with peptides containing valine at position 12 to detect unbound DWP by ELISA. Our results demonstrated that peptides containing glycine, alanine, serine, arginine, aspartic acid, or glutamic acid at position 12 did not bind DWP. In contrast, peptides containing valine at position 12, and to a lesser degree (5–10 X) peptides containing cysteine at position 12, were able to bind DWP.

We next tested the ability of DWP to bind normal or activated cellular ras proteins contained in NIH cells. Several attempts at immunoprecipitating p21 with DWP were unsuccessful and therefore we employed the Western blot procedure. To do this we initially concentrated the ras proteins from cell extracts by immunoprecipitation with the broadly crossreactive anti-ras MOAb Y13-259. Immunoprecipitates were electrophoresed in 12.5% polyacrylamide gels, transferred to nitrocellulose, and tested for reactivity with the positive control Y13-259, the negative control MOPC 141 or DWP. Cell lysates were derived from either NIH cells containing ras proteins with glycine at position 12 or NIH cells transformed by activated ras^H genes encoding valine at position 12. Results illustrated in Figure 1 show that ras proteins could be identified with the positive control MOAb Y13-259 in cells containing either normal ras proteins (lanes 1, 3) or mutated ras proteins containing valine at position 12 (lane 2,4). Results in Figure 1 show that the migration of the mutated p21 (valine-12) was found to migrate more slowly than normal ras p21 (glycine-12) in accordance with previously published results [24]. DWP reacted with ras proteins containing valine at position 12 (lanes 6,8) but not with normal ras proteins containing glycine at position 12 (lanes 5,7). No specific bands were observed in the p21 range when the MOPC 141 myeloma protein was substituted for DWP (lanes 9–12). Figure 1 also demonstrates that lysates from tissue culture cells (lanes 1, 2, 5, 6) gave results comparable to lysates prepared from nude mouse tumor cells (lanes 3, 4, 7, 8). The immunoblot experiment ilustrated in Figure 2 demonstrates that the positive control Y13-259 was reactive with p21s of all cell lines studied. DWP did not react with p21s from NIH cells (glycine-12) (panel A) but did react with NIH cells transformed by the ras^K gene from SW480 colon carcinoma cells encoding valine at position 12 of the p21 (panel

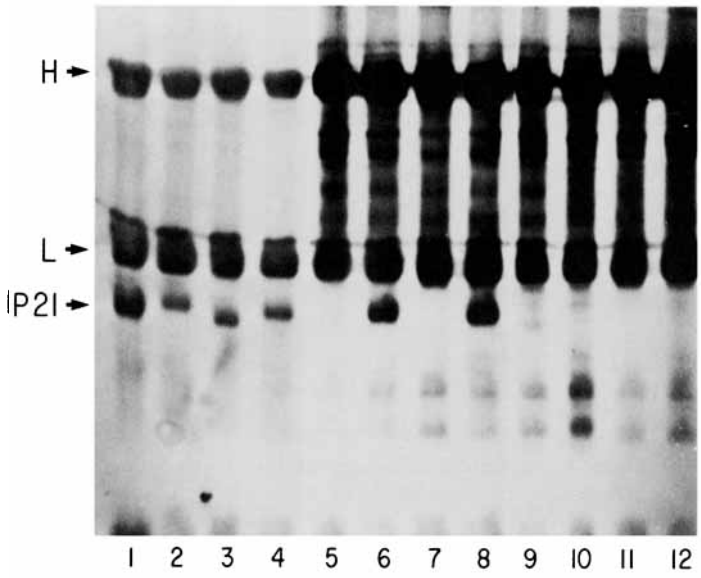

Fig. 1. P21 proteins were concentrated from cell extracts by immunoprecipitation with a broadly crossreactive rat MOAb Y13-259. NIH cells were transformed by either overexpression of the normal human ras^H gene (glycine at position 12) (lanes 1, 3, 5, 7, 9, and 11), or by the activated ras^H gene (valine at position 12) (lanes 2, 4, 6, 8, 10, and 12). Lanes 1, 2, 5, 6, 9, and 10 represent cell extracts derived from tissue culture cells whereas lanes 3, 4, 7, 8, 11, and 12 represent cell extracts from solid tumors produced in nude mice. Immunoprecipitates were collected, electrophoresed in 12.5% SDS-PAGE, and transferred to nitrocellulose filters for Western blot analysis. Filters were incubated with a 25 μg/ml of Y13-259 (lanes 1–4), DWP (lanes 5–8), or MOPC 141 (lanes 9–12) for 1 hr. Y13-259 was detected with rabbit anti-rat horseradish peroxidase (Cooper Biomedical Laboratories, Malvern PA), whereas DWP and MOPC 141 were detected with goat anti-mouse horseradish peroxidase (Bio-Rad, Richmond, CA). The p21 protein band is designated by an arrow. The light (L) and heavy (H) chains of immunoglobulins were also reactive with HRP reagents.

B). DWP did not react, however, with activated cellular p21s containing aspartic acid, glutamic acid, arginine, or serine at position 12 (panels C, D, E, and F). Similarly DWP did not react with ras proteins from transformed NIH lines containing cysteine at position 12 (data not shown).

Immunohistochemical analysis of cell lines containing ras proteins with glycine or valine at position 12 were also performed. Prior to incubation with DWP or with the negative control antibody MOPC 141, cells were formalin fixed and embedded in paraffin. Results were consistent with Western blots since positive immunoperoxidase staining as observed with transformed NIH cells containing activated p21s with valine at position 12. NIH cells containing normal p21 (glycine) or activated p21s encoding aspartic acid, glutamic acid, arginine, or serine at position 12 were not reactive by immunoperoxidase staining. An exception was found, however, in that NIH cells transformed by activated by p21s containing cysteine at position 12 were positive by immunoperoxidase.

DWP was also evaluated by Western blot and immunoperoxidase procedures on human bladder carcinoma cell line T24 and human lung adenocarcinoma cell line A549. Figure 3 shows a ras p21 band with the control MOAb Y13-259 in both cell lines. The bladder carcinoma T24 containing the activated ras^H gene and encoding

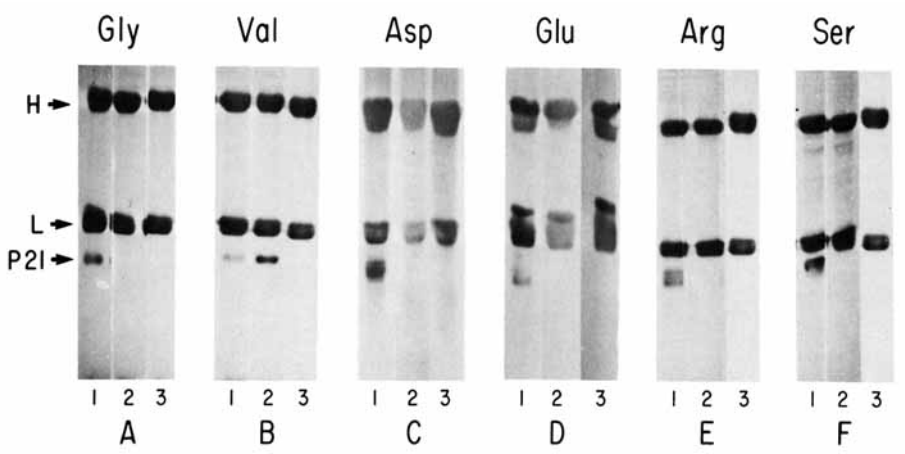

Fig. 2. P21 proteins were concentrated from cell extracts by immunoprecipitation with Y13-259. Cell extracts were derived from NIH cells transformed by overexpression of the normal human ras^H gene (glycine at position 12) (panel A), by an activated ras^H gene (valine at position 12) (panel B), by an activated ras^H gene from the HS0578t carcinosarcoma cell line (aspartic acid at position 12) (panel C), by an activated ras^H gene (glutamic acid at position 12) (panel D), by the viral ras^H gene (arginine at position 12) (panel E), or by the viral ras^K gene (serine at position 12) (panel F). Immunoprecipitates were collected, electrophoresed in 12.5% SDS-PAGE, and transferred to nitrocellulose filters for Western blot analysis. Filters were incubated with 25 μg/ml of Y13-259 (lane 1), DWP (lane 2), or MOPC 141 (lane 3) for 1 hr. Primary antibodies Y13-259, DWP, and MOPC 141 were detected as described in Figure 1.

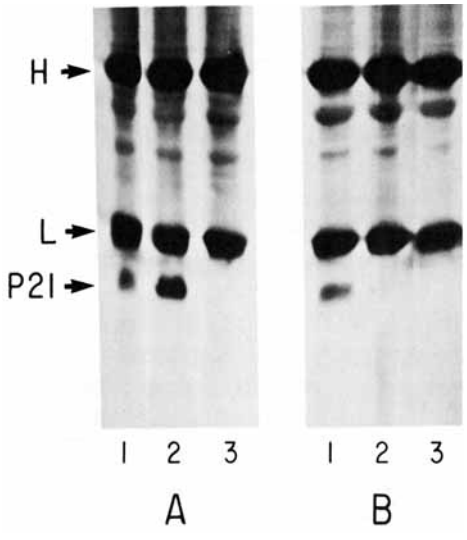

Fig. 3. P21 proteins were concentrated from human tumor cell line extracts T24 bladder carcinoma (panel A) and A549 lung adenocarcinoma (panel B) by immunoprecipitation with Y13-259. Immunoprecipitates were collected, electrophoresed in 12.5% SDS-PAGE, and transferred to nitrocellulose filters for Western blot analysis. Filters were incubated with 25μg/ml of Y13-259 (lane 1), DWP (lane 2), and MOPC 141 (lane 3) for 1 hr. Y13-259, DWP, and MOPC 141 were detected as described in Figure 1.

valine at position 12 was positive with DWP, whereas cell line A549 previously shown not to contain activated p21s [24] was not reactive with DWP. Similarly, human tumor cell lines SW1271 [27] and SK-N-SH [28] containing activated N-ras genes and having position 61 mutations were not reactive with DWP (data not shown).

DISCUSSION

Previous reports have described a rat MOAb, designated Y13-259 that cross-reacts with normal and mutated ras proteins in both yeast and mammalian cells. This antibody has been used to detect elevated levels of ras proteins in colon carcinoma cells. However, because Y13-259 is broadly cross-reactive, it could not be determined whether the elevated level of ras in the colon cells was due to activated or normal ras proteins [29]. MOAbs designated RAP have been shown to react in immunohistochemical staining with a high percentage of breast and colon carcinomas, but this antibody has also been reported to cross-react with normal and mutated forms of the ras proteins [30]. An antibody generated to ras-related proteins in rabbits against serine at position 12 has been shown to inhibit GTP binding to ras proteins. However, this antibody has not yet been evaluated for clinical utility [31].

Our objective in this study was to develop an antibody that would specifically react with activated ras proteins containing valine at position 12. In this report we describe a MOAb, DWP, that was raised against a synthetic peptide corresponding to positions 5–16 of the ras protein in T24 bladder carcinoma cells and that specifically reacts by Western blot and immunoperoxidase studies with activated ras proteins containing valine at position 12. DWP did not react by Western blot with normal p21s or activated p21s containing arginine, serine, glutamic acid, aspartic acid, or cysteine at position 12. Immunoperoxidase studies demonstrated, however, that DWP reacted with cells containing ras proteins with valine or cysteine at position 12.

Thus, in human tumors, DWP may allow one to determine the presence and frequency of activated p21s with valine at position 12, a point that we are currently evaluating. DWP and additional antibodies with specificities for activated ras proteins may be valuable in assessing the role of mutated ras in human malignancy.

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