RAPID COMMUNICATION Jpn. J. Cancer Res. 80, 10–14, January 1989

A Murine Monoclonal Antibody that Recognizes an Extracellular Domain of the Human c-*erbB*-2 Protooncogene Product

Takashi Masuko,[1] Kunio Sugahara,[1] Mitsuko Kozono,[1] Seiko Otsuki,[1] Tetsu Akiyama,[2] Tadashi Yamamoto,[3] Kumao Toyoshima[3] and Yoshiyuki Hashimoto[1,4]

[1]*Department of Hygienic Chemistry, Pharmaceutical Institute, Tohoku University, Aobayama, Sendai 980,* [2]*Institute for Virus Research, Kyoto University, Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606 and* [3]*Department of Oncology, Institute of Medical Science, Tokyo University, 4-6-1 Shiroganedai, Minato-ku, Tokyo 108*

A murine IgM monoclonal antibody, designated SV2-61, was generated against human c-*erbB*-2 gene-transfected NIH-3T3 (SV11) cells. SV2-61 defined a 185-kDa molecule present on the surface of SV11 cells, another line of c-*erbB*-2 gene-transfected NIH-3T3 (A4-15) cells, and MKN-7 human gastric cancer cell line carrying an amplified human c-*erbB*-2 gene. The SV2-61-defined antigen was found to show protein kinase activity *in vitro*. The SV2-61 was reactive with human c-*erbB*-2 gene-transfected NIH-3T3 cell lines but not with transfectants carrying c-*erbB*-2 gene mutants which lack a coding region for the extracellular domain. It was reactive with a portion of human epithelial cell lines but not with native NIH-3T3, TGF-α-coding gene-, activated c-*raf* gene- or Ha-*ras* gene-transfected NIH-3T3 cells, or non-epithelial human cells. These results indicate that the SV2-61 is an antibody which recognizes an extracellular domain of the c-*erbB*-2 gene product, 185-kDa protein.

Key words: Anti-c-*erbB*-2 protein monoclonal antibody — c-*erbB*-2 transfectants — Autophosphorylation

The c-*erbB*-2 gene is a protooncogene related to, but distinct from c-*erbB*-1, which encodes the epidermal growth factor receptor (EGF-R).[1] The protein encoded by human c-*erbB*-2 shows a close similarity with EGF-R and consists of extracellular, transmembrane and intracellular domains with tyrosine kinase activity.[2,3] Since this protooncogene is reported to be amplified in a variety of human adenocarcinomas,[1,4-8] it is likely that its product is also increased in these tumors. Therefore, derivation of antibodies reactive with the human c-*erbB*-2 gene product will be useful not only for characterization of the c-*erbB*-2 gene product but also for diagnosis and, probably, for therapy of certain human tumors. For these reasons, polyclonal antibodies reactive with the c-*erbB*-2 product have been prepared by immunizing rabbits with a synthetic polypeptide corresponding to a part of the protein sequence encoded by the human c-*erbB*-2[3,9] and used for identification of the human c-*erbB*-2 protein and its distribution.[10] However, these antibodies recognize an intracellular domain of the human c-*erbB*-2 gene product, but not an extracellular domain. This report describes the preparation of a monoclonal antibody (MoAb), which is reactive with the 185-kDa c-*erbB*-2 protein expressed on c-*erbB*-2 gene-transfected NIH-3T3 cells and some human cancer cell lines, and recognizes an extracellular domain of the product.

Hybridomas were established according to a standard method[11] by a somatic fusion between P3X63Ag8.653 mouse myeloma cells and spleen cells of BALB/c mice hyperimmunized against SV11 cells in which the full length c-*erbB*-2 cDNA is expressed under SV40 promoter. By screening of supernatants of hybridomas grown in about 3,000 wells of culture plates containing serum-free medium,[12] the hybridoma cells in one well were found to produce an antibody reactive with SV11 cells but not (or only weakly) reactive with NIH-3T3 cells. They were cloned by a limiting dilution method and expanded in serum-free medium using P3X63Ag8.653 cells as a feeder layer. The isotype of the monoclonal antibody produced from the clone was IgM, and it was designated SV2-61. A large quantity of SV2-61 MoAb was obtained from ascites of BALB/c mice which had received an ip inoculation of the SV2-61-hybridoma after pristane treatment. For immunofluorescence analysis, the MoAb was purified by ammonium sulfate precipitation followed by Sephacryl S-300 column chromatography and then diluted with phosphate-buffered saline, pH 7.4, containing 1% bovine serum albumin at a concentration of 10 μg/ml. The reactivity of SV2-61 MoAb with living target cells was determined by either regular indirect

[4] To whom reprint requests should be addressed.

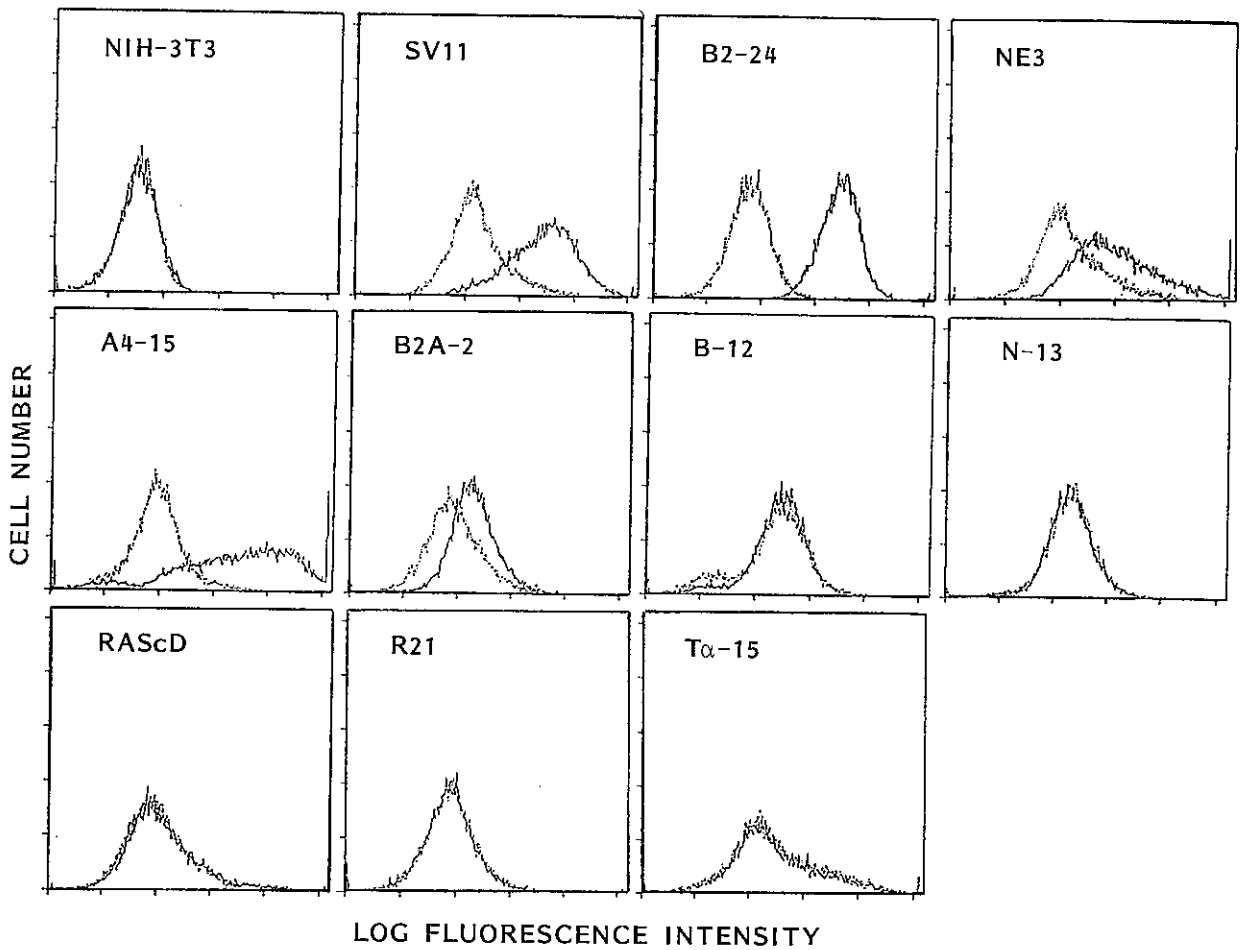

Fig. 1. Expression of SV2-61 MoAb-defined antigen on NIH-3T3 and its oncogene transformants. A cell suspension containing 1×10^6 test cells was successively treated with SV2-61 MoAb (1 μg in 100 μl) and a 1:200 dilution of FITC-conjugated rabbit antimouse immunoglobulins for 1 h at 4°C. Control samples were treated without the MoAb. Cell surface immunofluorescence of individual cells was determined by means of flow cytometry with the use of a FACS analyzer. Data for control and the SV2-61-stained cells are shown with dotted curves and solid curves (to the right of, or overlapping with control curves), respectively. Upper row, native NIH-3T3 cells and normal c-*erbB*-2 cDNA transfectants (SV11, B2-24 and NE3); middle row, NIH-3T3 cells transfected with mutated c-*erbB*-2 cDNA (A4-15 and B2A-2, c-*erbB*-2 gene with a point mutation; B-12 and N-13, c-*erbB*-2 with deletions at an extracellular domain coding region); lower row, NIH-3T3 cells transfected with oncogenes other than c-*erbB*-2 (RAScD, EJ*ras* gene; R21, activated c-*raf* gene; Tα-15, TGF-α gene).

membrane immunofluorescence or flow cytometry with use of a FACS analyzer (Becton Dickinson, Sunnyvale, CA).

To establish whether the SV2-61-defined antigen is related to c-*erbB*-2 gene expression, we examined the reactivity of the SV2-61 MoAb with oncogene-transfected and untransfected NIH-3T3 cells by means of flow cytometry (Fig. 1). The SV2-61 MoAb reacted with all three NIH-3T3-derived cell lines that had been transfected with the c-*erbB*-2 cDNA with SV40 promoter (SV11), Harvey sarcoma virus promoter (B2-24) or mouse tumor virus promoter (NE3), but not with native NIH-3T3 cells. It was also reactive with A4-15 and B2A-2 cell lines, which had been derived from NIH-3T3 cells transfected with an activated c-*erbB*-2, a *neu* type gene encoding glu^{659} instead of val^{659} at the transmembrane region,[13] under an SV40 promoter and a Harvey sarcoma virus promoter, respectively. By contrast, the SV2-61 MoAb was unreactive with B-12 and N-13 cell lines which had been prepared by transfection of NIH-3T3 with an SV40 promoter-primed c-*erbB*-2 mutant, which lacks a coding region for an extracellular domain of the

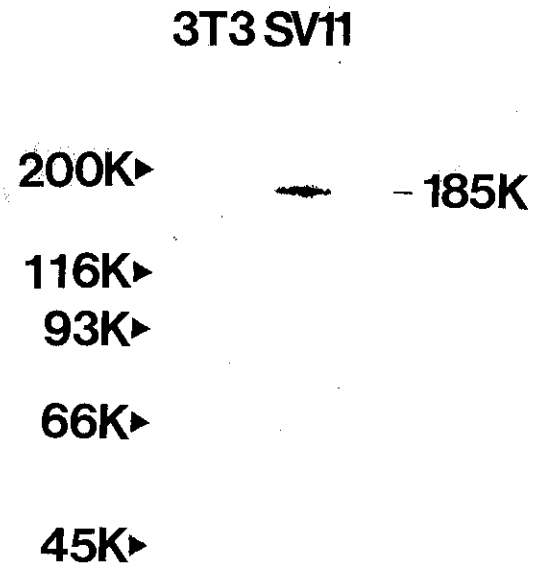

Fig. 2. Immunoprecipitation of an SV2-61 MoAb-defined antigen. An extract from ^{125}I surface-labeled SV11 cells was immunoprecipitated with SV2-61 MoAb. The precipitates were analyzed by SDS-PAGE (7.5%) under reducing conditions followed by autoradiography.

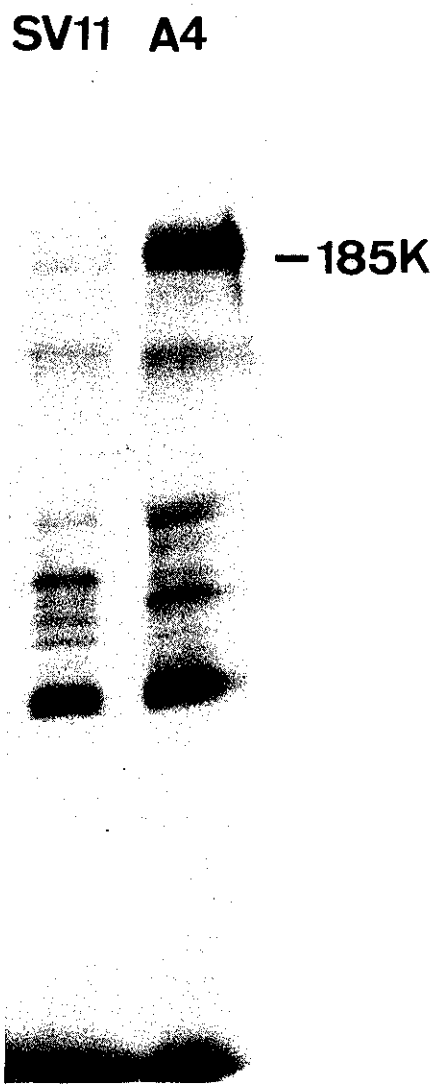

Fig. 3. Autophosphorylation of an SV2-61-defined antigen. Materials immunoprecipitated from SV11 (normal c-*erbB*-2 transfectant) or A4-15 (activated c-*erbB*-2 transfectant) cells with SV2-61 MoAb were treated with [γ-^{32}P]ATP and analyzed by SDS-PAGE followed by autoradiography as described by Akiyama *et al.*[3] Note a phosphorylated component at a molecular weight of 185 kDa, which corresponds to the c-*erbB*-2 protein.

c-*erbB*-2 product (No. 148-369 amino acids deletion in B-12 and No. 293-439 deletion in N-13). None of the cell lines obtained by transfection of NIH-3T3 cells with an oncogene other than c-*erbB*-2, such as EJ *ras*, activated c-*raf* or the TGF-α-coding gene, was reactive with the SV2-61 MoAb. Reactivity of the SV2-61 MoAb to cells bearing the SV2-61-defined antigen was diminished by heat treatment but not by $NaIO_4$ treatment. To determine the molecular weight of the SV2-61 MoAb-defined antigen, extracts from ^{125}I surface-labeled cells were immunoprecipitated with the SV2-61 MoAb and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The c-*erbB*-2 product having a molecular weight of about 185 kDa was immunoprecipitated from SV11 cells but not from native NIH-3T3 cells (Fig. 2). A cell surface component of 185 kDa was also immunoprecipitated from the extracts of the amplified c-*erbB*-2 gene-expressing MKN-7 human gastric cancer cells.[14] It is known that the c-*erbB*-2 protein posesses tyrosine kinase activity and is autophosphorylated *in vitro*,[3] and that the activity of the protein encoded by the activated c-*erbB*-2 gene is elevated as compared to that of the normal c-*erbB*-2 protein (Akiyama *et al.*, unpublished data). Thus, materials immunoprecipitated from c-*erbB*-2 transfectants with the SV2-61 MoAb were examined for their autophosphorylation activity using [γ-^{32}P]ATP as a marker. An activated-c-*erbB*-2 transfectant, A4-15 cells, afforded a highly phosphorylated component at the position of the c-*erbB*-2 protein (about 185 kDa), although little autophosphorylation was observed at this position with the material from SV11 cells (Fig. 3).

When the polyclonal antibody against c-*erbB*-2 protein[3] was used, the phosphorylation of the material immunoprecipitated from A4-15 cells was much weaker than that observed with the use of the SV2-61 MoAb (data not shown). This is probably due to binding of the polyclonal antibody close to the autophosphorylation site on an intracellular domain in the c-*erbB*-2 protein. Together with the cell membrane reactivity of the SV2-61 MoAb, these results indicate that the SV2-61 MoAb recognizes a protein epitope present on an extracellular domain of the c-*erbB*-2 gene product.

To determine the expression of the c-*erbB*-2 gene product on human tumor cells, we examined the reactivity of the SV2-61 MoAb with various human tumor cell lines by means of indirect membrane immunofluorescence and FACS analysis. In addition, these human cell lines were examined for the expression of the epidermal growth factor receptor (EGF-R) using an anti-EGF-R MoAb, 29-1,[15] because the c-*erbB*-2 product resembles EGF-R in protein sequence.[2] We tested a total of 25 human tumor cell lines and fetal fibroblasts. The SV2-61 MoAb was reactive with a portion of epithelial tumor cell lines including MKN-7 stomach cancer, SW116 colon cancer and HepG2 liver cancer cell lines, but not with any non-epithelial tumor cell lines such as SK-MEL-37 melanoma, SK-MG-1 glioma, Molt-4F T cell leukemia, Daudi B cell leukemia and K562 erythroleukemia cell lines and fetal fibroblasts (detail of the results will be reported elsewhere). It is notable that expressions of the SV2-61 MoAb-defined antigen (c-*erbB*-2 gene product) and the 29-1 MoAb-defined antigen (EGF-R) are generally different. For example, MKN-7 stomach cancer cells carrying the amplified c-*erbB*-2 gene[14] were found to react with the SV2-61 MoAb but not with the 29-1 MoAb. By contrast, A431 vulva cancer cells carrying amplified EGF-R gene[16] showed an opposite staining pattern.

In this work we prepared a MoAb which recognizes an extracellular domain of the human c-*erbB*-2 protein. Although a MoAb against a product of *neu* oncogene, a rat homologue of human c-*erbB*-2, has already been prepared[17] and shown to inhibit the growth of *neu*-transformed rat cells both *in vitro*[18] and *in vivo*,[19] this MoAb is not cross-reactive with the human c-*erbB*-2 gene product. Therefore, the SV2-61 MoAb will be useful not only for studies on functional analysis of the c-*erbB*-2 protein in the human system but also for clinical diagnosis and, probably, therapy of c-*erbB*-2-expressing human tumors.

This work was supported in part by a Grant-in-Aid for Special Project Research, Cancer Bioscience (No. 62615402), from the Ministry of Education, Science and Culture of Japan.

(Received October 7, 1988/Accepted November 29, 1988)

REFERENCES

1) Semba, K., Kamata, N., Toyoshima, K. and Yamamoto, T. A v-*erbB*-related protooncogene, c-*erbB*-2, is distinct from the c-*erbB*-1/epidermal growth factor-receptor gene and is amplified in a human salivary grand adenocarcinoma. *Proc. Natl. Acad. Sci. USA*, **82**, 6497–6501 (1985).
2) Yamamoto, T., Ikawa, S., Akiyama, T., Semba, K., Nomura, N., Miyajima, N., Saito, T. and Toyoshima, K. Similarity of protein encoded by the human c-*erbB*-2 gene to epidermal growth factor receptor. *Nature*, **319**, 230–234 (1986).
3) Akiyama, T., Sudo, C., Ogawara, H., Toyoshima, K. and Yamamoto, T. The product of the human c-*erbB*-2 gene: a 185-kilodalton glycoprotein with tyrosine kinase activity. *Science*, **232**, 1644–1646 (1986).
4) King, C. R., Kraus, M. H. and Aaronson, S. A. Amplification of a novel v-*erb*B-related gene in a human mammary carcinoma. *Science*, **229**, 974–976 (1985).
5) Yokota, J., Yamamoto, T., Toyoshima, K., Terada, M., Sugimura, T., Battifora, H. and Cline, M. J. Frequent amplification of the c-*erbB*-2 oncogene in human adenocarcinomas. *Lancet*, **i**, 765–767 (1986).
6) Zhou, D., Battifora, H., Yokota, J., Yamamoto, T. and Cline, M. J. Association of multiple copies of the c-*erbB*-2 oncogene with spread of breast cancer. *Cancer Res.*, **47**, 6123–6125 (1987).
7) Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullich, A. and McGuire, W. L. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. *Science*, **235**, 177–182 (1987).
8) Tal, M., Wetzler, M., Josefberg, Z., Deutch, A., Gutman, M., Assaf, D., Kris, R., Shiloh, Y., Givol, D. and Schlessinger, J. Sporadic amplification of the *HER2/neu* protooncogene in adenocarcinomas of various tissues. *Cancer Res.*, **48**, 1517–1520 (1988).
9) Gullick, W., Berger, M. S., Bennett, P. L. P., Rothbard, J. B. and Waterfield, M. D. Expression of the c-*erbB*-2 protein in normal and transformed cells. *Int. J. Cancer*, **40**, 246–254 (1987).
10) Mori, S., Akiyama, T., Morishita, Y., Shimizu, S., Sakai, K., Sudoh, K., Toyoshima, K. and Yamamoto, T. Light- and electron-microscopical demonstration of c-*erbB*-2 gene product-like immunoreactivity on human malignancy. *Virchow Arch.*, **54**, 8–15 (1987).
11) Masuko, T., Yagita, H. and Hashimoto, Y. Monoclonal antibodies against cell surface antigens present on human bladder cancer cells. *J. Natl. Cancer Inst.*, **72**, 523–530 (1984).
12) Yabe, N., Kato, M., Matsuya, Y., Yamane, I., Iizuka, M.,

Takayoshi, H. and Suzuki, K. Role of iron chelators in growth-promoting effect on mouse hybridoma cells in a chemically defined medium. *In Vitro Cell. Dev. Biol.*, **23**, 815–820 (1987).
13) Bargmann, C. I., Hung, M-C. and Weinberg, R. A. Multiple independent activations of the *neu* oncogene by a point mutation altering the transmembrane domain of p185. *Cell*, **45**, 649–657 (1986).
14) Fukushige, S., Matsubara, K., Yoshida, M., Sasaki, M., Suzuki, T., Semba, K., Toyoshima, K. and Yamamoto, T. Localization of a v-*erbB*-related gene, c-*erbB*-2, on the human chromosome and its amplification in MKN-7 gastric cancer cell line. *Mol. Cell. Biol.*, **6**, 955–958 (1986).
15) Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M. D. Close similarity of epidermal growth factor receptor and v-*erb-B* oncogene protein sequence. *Nature*, **307**, 521–527 (1984).
16) Xu, Y-H., Richert, N., Ito, S., Merlino, G. T. and Pastan, I. Characterization of epidermal growth factor receptor gene expression in malignant and normal human cell lines. *Proc. Natl. Acad. Sci. USA*, **81**, 7308–7312 (1984).
17) Drebin, J. A., Stern, D. F., Link, V. C., Weinberg, R. A. and Green, M. I. Monoclonal antibodies identify a cell-surface antigen associated with an activated cellular oncogene. *Nature*, **312**, 545–548 (1984).
18) Drebin, J. A., Link, V. C., Stern, D. F., Weinberg, R. A. and Green, M. I. Down-modulation of an oncogene protein product and reversion of the transformed phenotype by monoclonal antibodies. *Cell*, **41**, 695–706 (1985).
19) Drebin, J. A., Link, V. C., Weinberg, R. A. and Green, M. I. Inhibition of tumor growth by a monoclonal antibody reactive with an oncogene-encoded tumor antigen. *Proc. Natl. Acad. Sci. USA*, **83**, 9129–9133 (1986).