Vol. 181, No. 1, 1991
November 27, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 416-422

THE RET ONCOGENE PRODUCTS ARE MEMBRANE-BOUND GLYCOPROTEINS PHOSPHORYLATED ON TYROSINE RESIDUES IN VIVO

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Received October 21, 1991

Summary: We identified *ret* oncogene products in NIH 3T3 cells transformed by the *ret* oncogene (NIH(ret)) and a cell line (Lym-ret) established from pre-B cell lymphoma which developed in Eμ-*ret* transgenic mice. Using the polyclonal antibody against the kinase domain of *ret*, two glycoproteins with apparent molecular weights of 100kd and 96kd were found in both cell lines, although the expression level of the 100kd protein was much higher than that of the 96kd protein. Cell fractionation experiments indicated that the 100kd protein was present predominantly in the membrane fraction while the 96kd protein was found in both membrane and cytosol fractions. Western blot analysis indicated that the 100kd *ret* protein was phosphorylated on tyrosine residues *in vivo*.

The *ret* oncogene was first detected by transfection of NIH 3T3 cells with human T cell lymphoma DNA (1). It was activated by DNA rearrangement of the *ret* proto-oncogene, which encodes a receptor-type tyrosine kinase, with a finger-containing gene (*rfp*) during the transfection assay (1-5). The sequence of *ret* cDNA derived from transformed NIH cells indicated that the first 315 amino acids of *rfp* were fused to the amino terminus of the 5' truncated *ret* proto-oncogene. Subsequently, Ishizaka *et al.* (6) and Koda *et al.* (7) reported different rearrangements of the *ret* proto-oncogene which also occurred during transfection.

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Abbreviation: PTYR, phosphotyrosine.

0006-291X/91 $1.50

Recently, a novel rearranged form of the *ret* proto-oncogene was found to exist in human thyroid papillary carcinomas (8,9). This rearrangement occurred *in vivo*, resulting in the fusion of the tyrosine kinase domain of the *ret* proto-oncogene with 5'-terminal sequence of the H4 gene. In addition, it turned out that this fusion gene was generated by an intrachromosomal rearrangement, because both the *ret* proto-oncogene and the H4 gene were mapped to chromosome 10q11.2 (10,11).

We reported that the *ret* proto-oncogene products are expressed as cell surface glycoproteins in neuroblastoma and leukemia cells (12). To study the mechanism of transformation, it is important to identify the *ret* oncogene product and its subcellular structure. In the present study, we characterized the *ret* oncogene products in transformed NIH cells and a cell line established from pre-B cell lymphoma which developed in transgenic mice carrying the *ret* oncogene (13). Here we show that the *ret* oncogene products are membrane-bound glycoproteins.

Materials and Methods

Cell lines

NIH 3T3 cells transformed by the *ret* oncogene (NIH(ret)) were previously described (1). Lym-ret cell line was established from pre-B cell lymphoma developed in transgenic mice carrying the *ret* oncogene under the control of mouse immunoglobulin enhancer (13) and maintained in RPMI 1640 supplemented with 10% fetal calf serum.

Antibodies

Polyclonal antibody against the tyrosine kinase domain of the *ret* proto-oncogene was developed as described previously (12). Anti-phosphotyrosine antibody was purified by affinity chromatography from the sera of the rabbits immunized with v-*abl*-encoded bacteria protein (14).

Western blotting

Total cell lysates were prepared from the cell lines as described previously (12). The lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose (Schleicher & Schuell, Germany) or polyvinylidene difluoride (PVDF) (Nihon Millipore Kogyo K.K., Yonezawa, Japan) membranes. Reaction with anti-*ret* antibody was examined by the avidin-biotin complex immunoperoxidase method (12). Color development was performed using POD immunostain set (Wako Pure Chemical Ind., Ltd., Osaka, Japan). In case anti-PTYR antibody was used as the first antibody, the membranes were probed with ^{125}I-protein A (ICN, Irvine, CA, U.S.A.).

Cell fractionation

NIH(ret) and Lym-ret cells were incubated in hypotonic buffer (10mM Tris-HCl (pH7.4), 5mM $MgCl_2$, 0.5mM sodium orthovanadate, 1mM phenylmethyl-sulfonyl fluoride) on ice for 20 min. The cells were collected by scraping and homogenized in a tight-fitting Dounce homogenizer with 20 strokes. The homogenate was centrifuged at low speed (2,000 rpm) for

5 min. The pellet was resuspended in the same buffer, homogenized and centrifuged again at 2,000 rpm for 5 min. The resulting pellet was referred to as the nuclear fraction. After the first centrifugation, the supernatant was centrifuged at 100,000 x g for 30 min to obtain the membrane pellet. The resulting supernatant was referred to as the cytosol fraction.

Immunoprecipitation

Cells were lysed with 1ml of RIPA buffer (20mM Tris-HCl (pH7.5), 150mM NaCl, 2mM EDTA, 1% NP-40, 1mM sodium orthovanadate, and 1mM phenylmethyl-sulfonyl fluoride) and the resulting lysates were incubated with protein G-sepharose (Pharmacia, Sweden) precoated with anti-PTYR antibody for 1.5 hr at 4 °C. The immunoprecipitates were collected by centrifugation, washed and prepared for electrophoresis as described previously (15).

Results

Identification of *ret* oncogene products

To identify *ret* oncogene products, a total cell lysate was prepared from NIH(ret) cells and reacted with polyclonal antibody against the kinase domain of the *ret* protein. Western blot analysis revealed that the antibody recognized a major 100kd protein and a minor 96kd protein in the cell lysate (Fig. 1). These proteins were not found in a lysate from NIH 3T3 cells. In addition, the antibody reacted with a 78kd band in the lysates from NIH 3T3 and NIH(ret) cells, suggesting that it may represent a product of a tyrosine kinase gene which shows homology to the *ret* protein.

We further examined the expression of the *ret* protein in a Lym-ret cell line established from pre-B cell lymphoma which developed in Eμ-*ret* transgenic mice. This cell line expressed *ret* mRNAs at a high level (data not shown). The antibody detected the same 100kd and 96kd proteins in the lysate from Lym-ret cells as in NIH(ret) cells (Fig. 1).

It was shown that the amino acid sequence of the *ret* oncogene contains three potential N-linked glycosylation sites (2,3). To investigate whether the *ret* oncogene products are glycosylated, NIH(ret) and Lym-ret cells were treated with tunicamycin (5 μg/ml) and used for Western blotting. As shown in Fig. 2, the antibody reacted with a 93kd protein in both of them, indicating that the 100kd and 96kd proteins are glycoproteins. In addition, a faint 98kd band was observed in the lysate from NIH(ret) cells. This band was detected even when the cells were treated with a high concentration of tunicamycin (30 μg/ml) (data not shown). It is expected that NIH(ret) cells express two types of the *ret* proteins of 801 and 843 amino acids which differ in their carboxy-termini as observed for the *ret* proto-oncogene products (16). Thus the 93kd and 98kd proteins could represent these two proteins, respectively. Since the expression level of the 98kd is low, the glycosylated form of this protein may be undetectable.

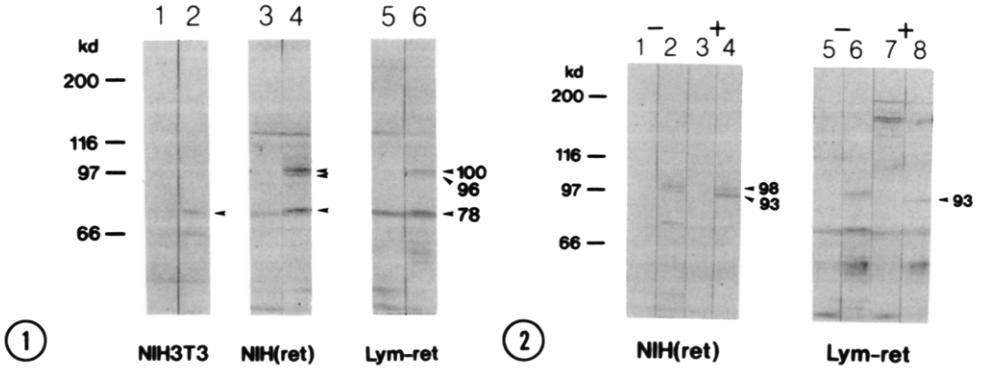

Fig. 1. Detection of the *ret* oncogene products in NIH(ret) and Lym-ret cells by Western blotting. The lysates containing 20 μg of proteins from NIH 3T3 (lanes 1 and 2), NIH(ret) (lanes 3 and 4) and Lym-ret (lanes 5 and 6) cells were separated on 8% SDS-polyacrylamide gels and analyzed by Western blotting. The antibodies used were normal rabbit IgG (10 μg/ml, lanes 1, 3 and 5) and anti-*ret* polyclonal antibody (10 μg/ml, lanes 2, 4 and 6). kd: kilodalton.

Fig. 2. Analysis of the *ret* oncogene products in tunicamycin-treated cells. Total cell lysates were prepared from NIH(ret) and Lym-ret cells which were cultured in the absence (lanes 1, 2, 5 and 6) or presence (lanes 3, 4, 7 and 8) of tunicamycin (5 μg/ml) and analyzed as described in Fig. 1. The antibodies used were normal rabbit IgG (10 μg/ml, lanes 1, 3, 5 and 7) and anti-*ret* polyclonal antibody (10 μg/ml, lanes 2, 4, 6 and 8).

Consistent with this view, only the 93kd protein was observed in the lysate from tunicamycin-treated Lym-ret cells in which the *ret* cDNA encoding the protein of 801 amino acids was expressed (Fig. 2).

To determine the subcellular localization of the *ret* oncogene products, NIH(ret) and Lym-ret cells were fractionated into nuclear, membrane and cytosol components. As shown in Fig. 3, the 100kd *ret* protein was detected predominantly in the membrane fraction. On the other hand, the 96kd protein was present in both membrane and cytosol fractions. Interestingly, we observed the 93kd protein corresponding to the unglycosylated form of the *ret* proteins in the cytosol fraction despite of the absence of this band in total cell lysates. In addition, the 110kd band detected in the membrane fraction could be non-specific because this band was also present in the membrane fraction of NIH 3T3 cells (data not shown).

Detection of PTYR-containing proteins

Using affinity purified anti-PTYR antibody, we examined the amounts of PTYR-containing proteins in the NIH(ret) and Lym-ret cells. Although the antibody was reactive with various sizes of bands in the lysates of NIH 3T3

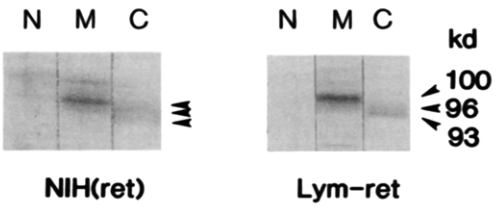

Fig. 3. Subcellular localization of the *ret* oncogene products. NIH(ret) and Lym-ret cells were fractionated into nuclear (N), membrane (M) and cytosol (C) fractions. The lysates (20 μg) from each fraction were reacted with anti-*ret* polyclonal antibody as described in Fig. 1.

and NIH(ret) cells, a band of approximately 100kd was specific in NIH(ret) cells (Fig. 4a). In the case of Lym-ret cells, several faint bands including 100, 115 and 120kd bands were detected. When NIH3T3 cells transformed by the *src* oncogene (NIH(src)) were examined, the amount of PTYR-containing protein was much higher than that in NIH(ret) or Lym-ret cells.

The 100kd band in the lysates of NIH(ret) and Lym-ret cells may be the autophosphorylated *ret* protein, because the electrophoretic mobility of this band was indistinguishable from that of the 100kd *ret* protein. To

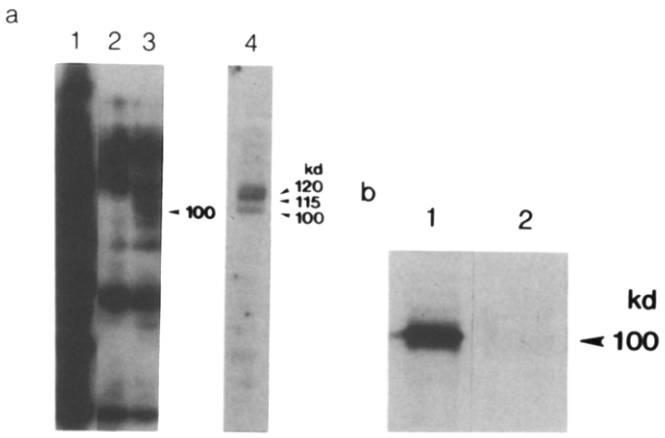

Fig. 4. Detection of phosphotyrosine-containing proteins. a. Cell lysates containing 50 μg of proteins were separated on 8% SDS-polyacrylamide gels and analyzed by Western blotting with anti-PTYR antibody (1 μg/ml). Lane 1: NIH(src) cells, lane 2: NIH 3T3 cells, lane 3: NIH(ret) cells, lane 4: Lym-ret cells. b. The lysate of Lym-ret cells immunopreciptated with anti-PTYR antibody was subjected to SDS-polyacrylamide gels and reacted with anti-*ret* antibody (lane 2). As a control, the total lysate of Lym-ret cells was reacted with the same antibody (lane 1).

confirm this, the lysates were immunoprecipitated with the anti-PTYR antibody and the resulting immunoprecipitates were reacted with the anti-*ret* antibody. As shown in Fig. 4b, the anti-*ret* antibody detected the 100kd band in the immunoprecipitates, indicating that it represents the phosphorylated *ret* protein. Although the 96 kd *ret* protein was undetectable by Western blotting with anti-PTYR antibody, it could be due to the low level of its expression.

Discussion

To determine the subcellular localization of oncogene products is important to study the mechanisms of cell transformation. In the present study, we showed that the *ret* oncogene products are expressed as two types of glycoproteins of 100kd and 96kd in NIH(ret) and Lym-ret cells. Cell fractionation experiments indicated that the 100kd protein was detected predominantly in the membrane fraction while the 96kd protein was present in both membrane and cytosol fractions. As reported for other transmembrane proteins (17-19), we suppose that the 100kd and 96kd proteins represent the mature and immature glycosylated forms of the *ret* proteins, respectively. Since the amino acid sequence of the *ret* cDNAs contains a transmembrane domain, it seems likely that the mature form of the *ret* oncogene products is a transmembrane protein at the plasma membrane. However, the immunohistochemical study could be necessary to identify definite localization of these proteins.

We investigated phosphotyrosine levels of cellular proteins in NIH(ret) and Lym-ret cells. In contrast to the results of NIH(src) cells, we were not able to detect high amounts of PTYR-containing proteins in NIH(ret) and Lym-ret cells. However, a 100kd tyrosine phosphorylated band was specifically detected in the lysates from both cells. The immunoprecipitation experiment indicated that this 100kd band represents the *ret* protein. Thus, the *ret* protein appears to be phosphorylated on tyrosine residues *in vivo*.

The mechanisms of transformation by tyrosine kinase oncogenes remain to be elucidated. To solve this problem, it is important to identify target proteins phosphorylated by the oncogene products which are involved in the transformation process. As mentioned above, however, no specific PTYR-containing band other than the *ret* protein was detected in NIH(ret) cells by Western blotting. In the case of Lym-ret cells, a few PTYR-containing proteins were found. These results indicated that target proteins phosphorylated by the *ret* protein are not so many as those phosphorylated by the *src* protein. Alternatively, target proteins of the *ret* protein which are necessary for cell transformation may be undetectable by Western

blotting. Since the *ret* proteins were found to localize in the membrane fraction, it could be important to investigate the functions of membrane-associated proteins which are involved in the intracytoplasmic signal transduction.

Acknowledgments

This work was supported in part by Grants-in-Aid for Cancer Research and for Scientific Research from the Ministry of Education, Science and Culture, Japan and by the Life Science Research Project of Institute of Physical and Chemical Research (RIKEN).

References

1. Takahashi, M., Ritz, J. and Cooper, G. M. (1985) Cell 42, 581-588.
2. Takahashi, M. and Cooper, G. M. (1987) Mol. Cell. Biol. 7, 1378-1385.
3. Takahashi, M., Inaguma, Y., Hiai, H. and Hirose, F. (1988) Mol. Cell. Biol. 8, 1853-1856.
4. Takahashi, M., Buma, Y., Iwamoto, T., Inaguma, Y., Ikeda, H. and Hiai, H. (1988) Oncogene 3, 571-578.
5. Takahashi, M., Buma, Y. and Hiai, H. (1989) Oncogene 4, 805-806.
6. Ishizaka, Y., Ochiai, M., Tahira, T., Sugimura, T. and Nagao, M. (1989) Oncogene 4, 789-794.
7. Koda, T. (1988) Hokkaido J. Med. Sci. 63, 913-924.
8. Grieco, M., Santoro, M., Teresa Berlingieri, M., Marina Melillo, R., Donghi, R., Bongarzone, I., Pierotti, M. A., Della Porta, G., Fusco, A., and Vecchio, G. (1990) Cell 60, 557-563.
9. Ishizaka, Y., Ushijima, T., Sugimura, T. and Nagao, M. (1990) Biochem. Biophys. Res. Commun. 168, 402-408.
10. Donghi, R., Sozzi, G., Pierotti, M. A., Biunno, I., Miozzo, M., Fusco, A., Grieco, M., Santoro, M., Vecchio, G., Spurr, N. K. and Della Porta, G. (1989) Oncogene 4, 521-523.
11. Ishizaka, Y., Itoh, F., Tahira, T., Ikeda, I., Sugimura, T., Tucker, J., Fertitta, A., Carrano, A. V. and Nagao, M. (1989) Oncogene 4, 1519-1521.
12. Takahashi, M., Buma, Y. and Taniguchi, M. (1991) Oncogene 6, 297-301.
13. Iwamoto, T., Pu, M., Ito, M., Takahashi, M., Isobe, K., Nagase, F., Kawashima, K., Ichihara, M. and Nakashima, I. (1991) Eur. J. Immunol. 21, 1809-1814.
14. Hamaguchi, M., Grandori, C. and Hanafusa, H. (1988) Mol. Cell. Biol. 8, 3035-3042.
15. Escobedo, J. A., Navankasattusas, S., Kavanaugh, W. M., Milfay, D., Fried, V. A. and Williams, L. T. (1991) Cell 65, 75-82.
16. Tahira, T., Ishizaka, Y., Itoh, F., Sugimura, T. and Nagao, M. (1990) Oncogene 5, 97-102.
17. Rettenmier, C. W., Roussel, M. F., Ashmun, R. A., Ralph, P., Price, K. and Sherr, C. J. (1987) Mol. Cell. Biol. 7, 2378-2387 (1987).
18. Martin-Zanca, D. Oskam, R., Mitra, G., Copeland, T and Barbacid, M. (1989) Mol. Cell. Biol. 9, 24-33.
19. Nocka, K., Tan, J. C., Chiu, E., Chu, T. Y., Ray, P., Traktman, P. and Besmer, P. (1990) EMBO J. 9, 1805-1813.