The Presence of c-*erb*B-2 Gene Product-related Protein in Culture Medium Conditioned by Breast Cancer Cell Line SK-BR-3[1]

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Abstract

The M_r 185,000 glycoprotein encoded by human c-*erb*B-2/*neu*/HER2 gene, termed c-*erb*B-2 gene product, shows a close structural similarity with epidermal growth factor receptor and is now regarded to be a growth factor receptor for an as yet unidentified ligand. Abundant c-*erb*B-2 mRNA was demonstrated by Northern blot studies in the human breast cancer cell line SK-BR-3. Cellular radiolabeling experiments followed by immunoprecipitation with three different anti-c-*erb*B-2 gene product antibodies, recognizing extracellular domain, kinase domain, and carboxyl-terminal portion, respectively, demonstrated the production of a large amount of c-*erb*B-2 gene product which had the capacity to be phosphorylated.

Immunization of mice with concentrated culture medium conditioned by SK-BR-3 cells always generated antibodies against c-*erb*B-2 gene product, demonstrating that this culture medium contained substance(s) immunologically indistinguishable from c-*erb*B-2 gene product. This observation was supported by the successful development of a monoclonal antibody against c-*erb*B-2 gene product, GFD-OA-p185-1, by immunizing mice with this culture medium.

The biochemical nature of the substance(s) present in the culture medium was further characterized. When the culture medium conditioned by [^{35}S]cysteine-labeled SK-BR-3 cells was immunoprecipitated by three different anti-c-*erb*B-2 gene product antibodies, only the antibody recognizing extracellular domain precipitated the [^{35}S]-labeled protein with a molecular weight of 110,000, namely p110. The newly developed monoclonal antibody also immunoprecipitated this protein. However, when the culture medium conditioned by ^{32}P-labeled SK-BR-3 cells was examined in the same manner, no labeled protein was immunoprecipitated by four anti-c-*erb*B-2 gene product antibodies. Thus, p110 may be the actual c-*erb*B-2 gene product-related protein present in the culture medium conditioned by SK-BR-3 cells; furthermore, p110 may have a structural similarity with that of the extracellular domain of c-*erb*B-2 gene product. The present study also demonstrates that immunization of mice with concentrated culture medium conditioned by A-431 cells generated antibodies against epidermal growth factor receptor. Thus, it is possible to postulate that culture medium conditioned by cancer cells could be a possible immunogen for developing an antibody against growth factor receptors.

Introduction

Two independent studies by Mayes and Waterfield (1) and by Weber *et al.* (2) demonstrated by experiments on EGFR[3] biosynthesis that a molecule with a structural similarity with the EGFR was secreted into the culture medium of A-431 cells, a cancer cell line overexpressing EGFR (3). Since this molecule was reported to have a molecular weight of approximately 105,000, possessed the capacity to bind epidermal growth factor, and contained the same amino acid sequences as the amino-terminal portion of EGFR, this molecule was designated EGFR-related protein (1, 2). Although these observations raised the concept of soluble growth factor receptors, or their related molecules, further studies on this EGFR-related protein revealed that this phenomenon was observed only in A-431 cells (4).

The M_r 185,000 glycoprotein encoded by the human c-*erb*B-2/*neu*/HER2 gene (5–7), designated as c-*erb*B-2 gene product, possesses a structure composed of a cysteine-rich extracellular domain, a transmembrane domain, and a highly conserved tyrosine kinase domain; it displays approximately a 50% homology in amino acid sequence to EGFR (7) and has the capacity to be phosphorylated at the tyrosine residue (8, 9). This protein is currently regarded as a growth factor receptor for an as yet unidentified ligand (10).

In the present study, we demonstrated that immunization of mice with concentrated culture medium conditioned by a breast cancer cell line expressing a large

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; MoAb, monoclonal antibody; PoAb, polyclonal antibody; NMS, normal mouse serum; FCS, fetal calf serum; ELISA, enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; kb, kilobase(s).

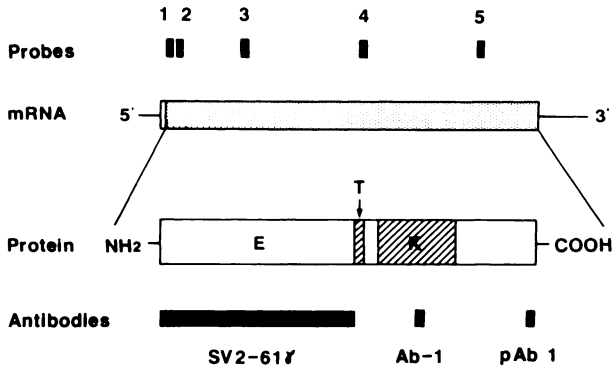

Fig. 1. Structure of c-*erb*B-2 mRNA and c-*erb*B-2 gene product. In the scheme of c-*erb*B-2 mRNA, the coding region and the untranslated region are shown by the *box* and the *solid line*, respectively. *Shaded portion*, the putative mature protein. *Black rectangles* above the mRNA structure, the portions of mRNA where the 5 probes can hybridize; *Probes 1, 2,* and *3* recognize the portions coding sequence for the extracellular domain of c-*erb*B-2 gene product (2–21, 34–52, and 254–273, respectively); *Probe 4* for the transmembrane domain of the protein (654–675); and *Probe 5* for the cytoplasmic domain of the protein (1055–1074). In the scheme of c-*erb*B-2 protein, three domains were demonstrated; these are extracellular (*E*), transmembrane (*T*), and kinase (*K*) domains. *Black rectangles* below the protein structure, the sites where the three antibodies, SV2-61γ, Ab-1, and pAb 1, can recognize.

amount of c-*erb*B-2 gene product, SK-BR-3 cells (11), always raised antibodies against this protein, indicating that this culture medium contained substance(s) immunologically indistinguishable from c-*erb*B-2 gene product. This observation was supported by the successful development of a MoAb against c-*erb*B-2 gene product by immunizing mice with this culture medium. Accordingly, we commenced to characterize the nature of this substance(s) present in the culture medium. The present study also indicates that culture medium conditioned by cancer cells is a potent immunogen for developing MoAb against growth factor receptors.

Results

Northern Blot Analysis in Cell Lines. SK-BR-3 cells and A-431 cells were known to express a large amount of growth factor receptor-like molecules; the former, c-*erb*B-2 gene product, and the latter, EGFR. Both of these cell lines were examined for expression of c-*erb*B-2 mRNA. Five synthetic oligodeoxyribonucleotide probes were designed to hybridize to the portions of c-*erb*B-2 mRNA corresponding to the extracellular, transmembrane, and cytoplasmic domains (Fig. 1). Autoradiography of Northern blot analysis using three probes for c-*erb*B-2 mRNA is shown in Fig. 2. In SK-BR-3 cells, when the probes hybridizable to the portion of c-*erb*B-2 mRNA corresponding to the extracellular domain were used (Fig. 2, *A* and *B*), two bands with molecular sizes of 5.0 and 2.8 kb were detected; the intensity of the former was stronger than that of the latter. With the probe hybridizable to the portion of c-*erb*B-2 mRNA corresponding to the transmembrane domain (Fig. 2*C*), only the band with a molecular size of 5.0 kb was detected. In A-431 cells, the band was not detected in the present experimental condition. When the probe for β-actin mRNA was used, a band corresponding to β-actin mRNA was detected in each cell line (Fig. 2*D*). Additionally, when Probe 3, hybridizable to the portion of c-*erb*B-2 mRNA corresponding to the extracellular domain, was used, a similar result was obtained to that of Probes 1 and 2; conversely, in the case of Probe 5, hybridizable to the portion of c-*erb*B-2 mRNA corresponding to the cytoplasmic domain, only the band with a molecular size of 5.0 kb was detected (data not shown).

^{32}P-labeled Protein in Cell Lines for Characterization of Antibodies. It is well established that c-*erb*B-2 gene product as well as EGFR have the capacity to be phosphorylated. Accordingly, ^{32}P-labeled proteins in cell lysates were examined by immunoprecipitation with antibodies against c-*erb*B-2 gene product or EGFR. In ^{32}P labeling studies of SK-BR-3 cells, two PoAbs recognizing kinase domain and carboxyl-terminal portion and one MoAb recognizing extracellular domain of c-*erb*B-2 gene product (Fig. 3*A*, *Lanes a–c*) immunoprecipitated the phosphorylated protein with a M_r 185,000 c-*erb*B-2 gene product. Anti-EGFR antibody recognizing extracellular domain of EGFR, as well as NMS, did not immunoprecipitate this protein (Fig. 3*A*, *Lanes e* and *f*). In ^{32}P labeling studies of A-431 cells, no phosphorylated band corresponding to c-*erb*B-2 gene product was detected with antibodies against c-*erb*B-2 gene product (Fig. 3*B*, *Lanes*

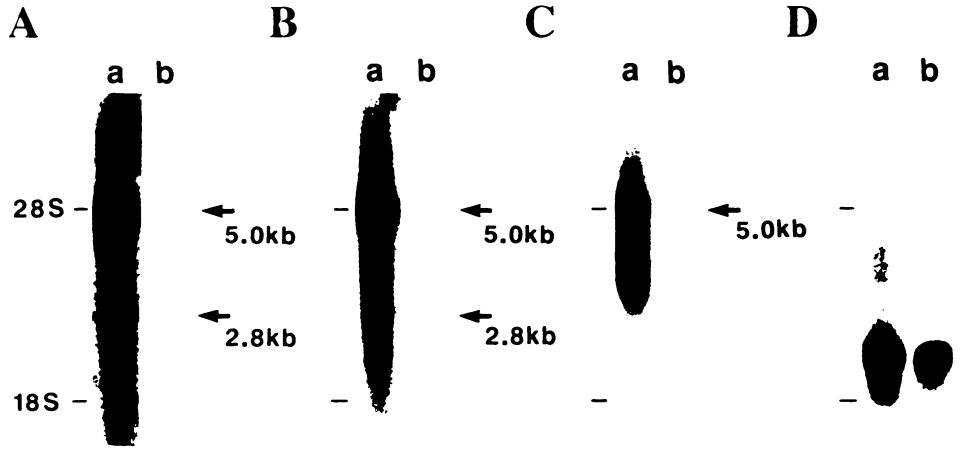

Fig. 2. Northern blot analyses using probes for c-*erb*B-2 mRNA and β-actin mRNA. *Lane a*, SK-BR-3 cells; *Lane b*, A-431 cells. *A*, Probe 1 for c-*erb*B-2 mRNA; *B*, Probe 2 for c-*erb*B-2 mRNA; *C*, Probe 4 for c-*erb*B-2 mRNA; *D*, probe for β-actin mRNA. *28S*, 28S ribosomal RNA; *18S*, 18S ribosomal RNA.

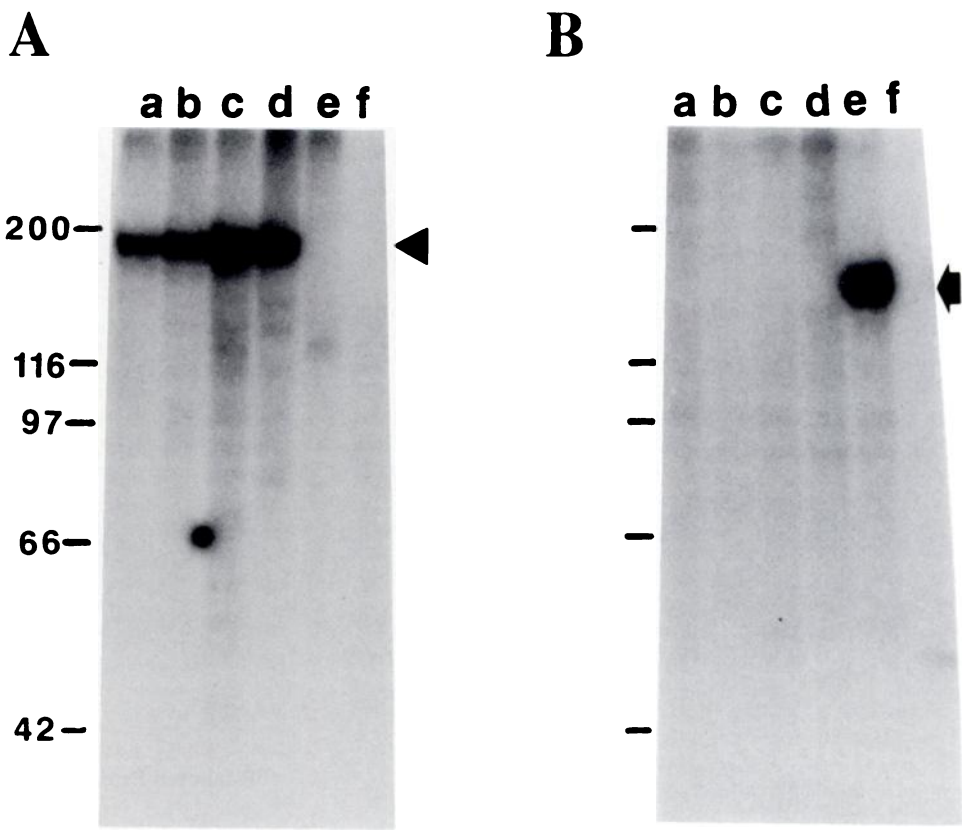

Fig. 3. Immunoprecipitation of ^{32}P-labeled c-*erb*B-2 gene product (◀) and EGFR (◖) from SK-BR-3 cell lysate (*A*) and A-431 cell lysate (*B*). Each *lane* indicates the results of immunoprecipitation by following antibodies: *Lane a*, PoAb against c-*erb*B-2 gene product (Ab-1); *Lane b*, PoAb against c-*erb*B-2 gene product (pAb 1); *Lane c*, MoAb against c-*erb*B-2 gene product (SV2-61γ); *Lane d*, newly developed MoAb against c-*erb*B-2 gene product (GFD-OA-p185-1); *Lane e*, MoAb against EGFR; and *Lane f*, NMS.

a–c), but phosphorylated EGFR with a molecular weight of 170,000 was immunoprecipitated by anti-EGFR antibody (Fig. 3*B*, *Lane* e).

Development of PoAb against c-*erb*B-2 Gene Product. Thirty mice were divided into five groups and were immunized with the following substances: Group 1, FCS alone; Group 2, 20-fold concentrated RPMI-1640 medium; Group 3, SK-BR-3 cells; Group 4, 20-fold concentrated culture medium conditioned by SK-BR-3 cells; and Group 5, 20-fold concentrated culture medium conditioned by A-431 cells.

PoAbs developed in mice were tested for the capacity to immunoprecipitate phosphorylated c-*erb*B-2 gene product in cell lysate of SK-BR-3 cells. No activity was detected in NMS (Fig. 4*A*, *Lane* c) and the sera obtained from mice given injections of FCS alone (Group 1; Fig.

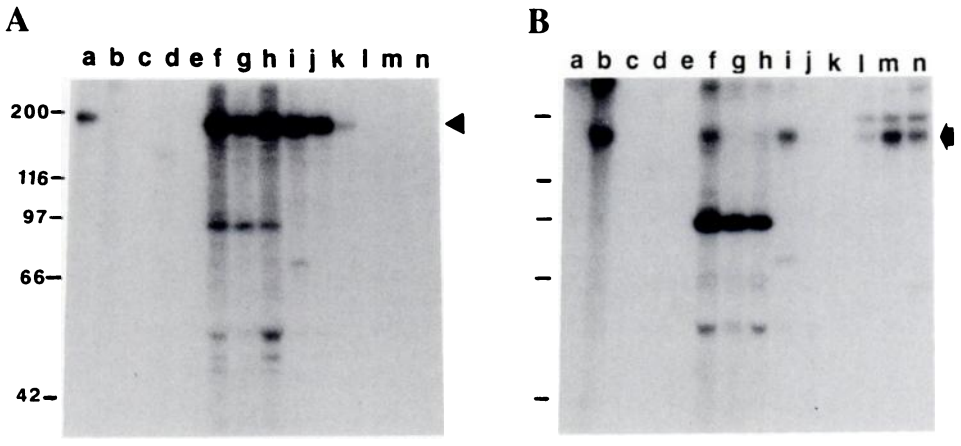

Fig. 4. Immunoprecipitation of ^{32}P-labeled c-*erb*B-2 gene product (◀) and EGFR (◖) from SK-BR-3 cell lysate (*A*) and A-431 cell lysate (*B*) by mouse antisera immunized with cancer cells and their culture media. *Lanes* represent the results of immunoprecipitation by the following sera: *Lane a*, MoAb against c-*erb*B-2 gene product (SV2-61γ); *Lane b*, MoAb against EGFR; *Lane c*, NMS; *Lane d*, antiserum immunized by FCS; *Lane e*, antiserum immunized by concentrated RPMI-1640; *Lanes f–h*, antiserum immunized by SK-BR-3 cells; *Lanes i–k*, antiserum immunized by concentrated culture medium conditioned by SK-BR-3 cells; *Lanes l–n*, antiserum immunized by concentrated culture medium conditioned by A-431 cells.

4A, *Lane d*), concentrated RPMI-1640 (Group 2; Fig. 4A, *Lane e*), and culture medium conditioned by A-431 cells (Group 5; Fig. 4A, *Lanes l–n*). Sera obtained from six mice that received injections of SK-BR-3 cells (Group 3) immunoprecipitated several phosphoproteins from cell lysate of ^{32}P-labeled SK-BR-3 cells (Fig. 4A, *Lanes f–h*); the most intense band had a molecular size of 185,000 and was regarded as an actual ^{32}P-labeled c-erbB-2 gene product, since the same band was immunoprecipitated with anti-c-erbB-2 gene product MoAb (Fig. 4A, *Lane a*). Meanwhile, sera obtained from 11 mice given injections of concentrated culture medium conditioned by SK-BR-3 cells (Group 4) specifically immunoprecipitated the phosphoprotein which identified as c-erbB-2 gene product. Fig. 4A (*Lanes i–k*) reveals representative results on the development of PoAb against c-erbB-2 gene product in mice.

When A-431 cells were phosphorylated and then examined, the mice receiving injections of concentrated culture medium conditioned by A-431 (Group 5) developed antibodies recognizing the band with a molecular weight of 170,000 (Fig. 4B, *Lanes l–n*); this band was regarded as phosphorylated EGFR according to the result using EGFR MoAb (Fig. 4B, *Lane b*). Sera obtained from this group also immunoprecipitated an additional band with a molecular size of 200,000; this band has not yet been characterized. It is worth noting that a band with a molecular size of 170,000 was detected in two PoAbs immunized with SK-BR-3 cells and culture medium conditioned by SK-BR-3 cells (Fig. 4B, *Lanes f* and *i*).

MoAb against c-erbB-2 Gene Product. MoAb against c-erbB-2 gene product was developed using mice immunized with concentrated culture medium conditioned by SK-BR-3 cells. Two mice with sera possessing a high titer of activity to immunoprecipitate phosphorylated c-erbB-2 gene product were used. Growth of hybrid cells was observed in almost all wells. From 774 wells, 17 hybrid cultures were selected that secreted mouse immunoglobulin recognizing membrane fraction-associated proteins by screening their media with an ELISA. Thereafter, the second screening was for activity to immunoprecipitate phosphorylated c-erbB-2 gene product. One well, containing the highest titer to immunoprecipitate the phosphorylated c-erbB-2 gene product, was further processed to a limited dilution, and the third limited dilution was successfully completed.

Immunoglobulins in 350 ml of culture media conditioned by the hybridoma clone characterized after the third limited dilution was first concentrated with ammonium sulfate precipitation and then purified with an affinity column using Affi-gel Protein A-agarose. The subtype of this MoAb was shown to be IgG1k and named GFD-OA-p185-1.

Characterization of Newly Developed MoAb. The newly developed MoAb, GFD-OA-p185-1, precipitated ^{32}P-labeled and ^{35}S-labeled c-erbB-2 gene product in cell lysate specifically (Fig. 3A, *Lane d*, and Fig. 5A, *Lane e*). When a ^{32}P-labeled A-431 cell lysate was used, no specific band was detected by GFD-OA-p185-1 (Fig. 3B, *Lane d*).

^{32}P-labeled and [^{35}S]Cysteine-labeled Proteins in Cell Lysate and Cultured Media. In the study using cell lysate of ^{32}P-labeled SK-BR-3 and A-431 cells, ^{32}P-labeled c-erbB-2 gene product was specifically immunoprecipitated by the anti-c-erbB-2 gene product antibodies rec-

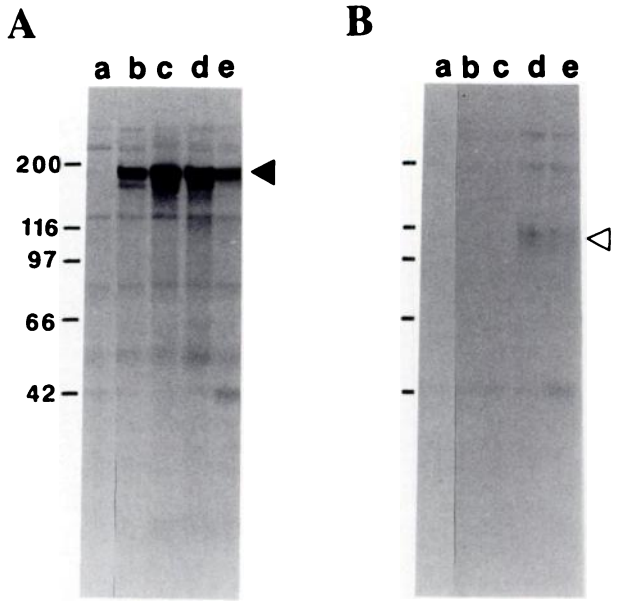

Fig. 5. Immunoprecipitation of [^{35}S]cysteine-labeled c-erbB-2 gene product (◀) and its related protein (◁) from cell lysate (*A*) and culture medium (*B*) of SK-BR-3 cells. *Lanes* represent the results of immunoprecipitation by the following antibodies: *Lane a*, NMS; *Lane b*, PoAb against c-erbB-2 gene product (Ab-1); *Lane c*, PoAb against c-erbB-2 gene product (pAb 1); *Lane d*, MoAb against c-erbB-2 gene product (SV2-61γ); and *Lane e*, newly developed MoAb against c-erbB-2 gene product (GFD-OA-p185-1).

ognizing extracellular domain, kinase domain, and carboxyl-terminal portion as well as by GFD-OA-p185-1 and ^{32}P-labeled EGFR, by the anti-EGFR antibody, as shown in Fig. 3. In contrast, when the culture media conditioned by ^{32}P-labeled SK-BR-3 and A-431 cells were examined, no specific band was immunoprecipitated with the four anti-c-erbB-2 gene product antibodies and one anti-EGFR antibody (data not shown).

In [^{35}S]cysteine labeling of SK-BR-3 cells, four different anti-c-erbB-2 gene product antibodies identified the band with a molecular size of 185,000 in cell lysate (Fig. 5A, *Lanes b–e*). Although several other nonspecific bands were detected by NMS (Fig. 5A, *Lane a*), NMS did not identify this band, indicating that this was [^{35}S]cysteine-labeled c-erbB-2 gene product. When the culture medium conditioned by [^{35}S]-labeled SK-BR-3 cells was examined, two PoAbs against c-erbB-2 gene product, recognizing kinase domain and carboxyl-terminal portion, respectively, did not show any specific band (Fig. 5B, *Lanes b* and c), when compared with the results using NMS (Fig. 5B, *Lane a*). However, the anti-c-erbB-2 gene product MoAb (SV2-61γ) recognizing the extracellular domain and the newly developed MoAb (GFD-OA-p185-1) immunoprecipitated a specific band with a molecular size of approximately 110,000 (Fig. 5B, *Lanes d* and e), suggesting that c-erbB-2 gene product-related protein was present in the culture medium conditioned by SK-BR-3 cells.

In order to compare this molecule with EGFR-related protein produced by A-431 cells, [^{35}S]cysteine-labeled SK-BR-3 and A-431 cells were further analyzed. With longer incubation of SK-BR-3 cells with [^{35}S]cysteine, the band immunoprecipitated with GFD-OA-p185-1 be-

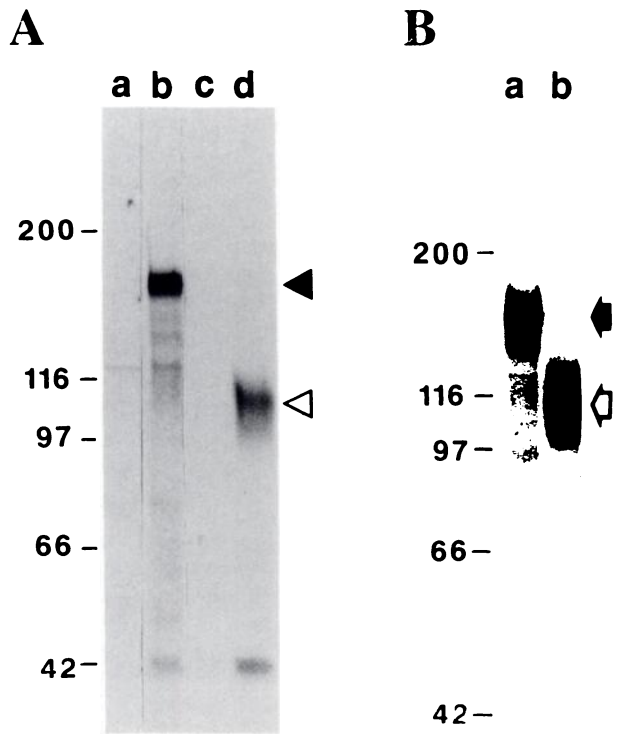

Fig. 6. Immunoprecipitation of [^{35}S]cysteine-labeled growth factor receptor-related proteins present in culture medium conditioned by SK-BR-3 cells (*A*) and A-431 cells (*B*). SDS-PAGE was performed with 5% polyacrylamide gel. Each *lane* indicates the results of the following immunoprecipitation. *A*: *Lane a*, cell lysate with NMS; *Lane b*, cell lysate with anti-c-*erb*B-2 gene product MoAb (GFD-OA-p185); *Lane c*, culture medium with NMS; *Lane d*, culture medium with anti-c-*erb*B-2 gene product MoAb (GFD-OA-p185). *B*: *Lane a*, cell lysate with anti-EGFR MoAb; *Lane b*, culture medium with anti-EGFR MoAb. ◀, c-*erb*B-2 gene product; ◁, c-*erb*B-2 gene product-related protein; ⧫, EGFR; ◊, EGFR-related protein.

came more clear (Fig. 6*A*, *Lane d*); by SDS-PAGE with 5% polyacrylamide gel, the molecular size of the specific band in the culture medium was calculated to be 110,000; accordingly, we tentatively termed this protein as p110. It is worth noting that the radioactivity of this band is not so much different when compared to that of c-*erb*B-2 gene product in cell lysate, suggesting that a large amount of p110 was produced and was secreted into culture medium. The same results were obtained using another anti-c-*erb*B-2 gene product MoAb, SV2-61γ, recognizing extracellular domain of c-*erb*B-2 gene product (data not shown). Regarding the band with a molecular weight of 42,000 (Fig. 6*A*, *Lane d*), it was also detected by NMS (Fig. 5, *A* and *B*, *Lane a*); therefore, we regarded it as nonspecific band, although its amount increased with the longer incubation time. When A-431 cells were examined, EGFR-related protein with a molecular size of 115,000 was detected (Fig. 6*B*, Lane b).

Discussion

The present study clearly demonstrates that a large amount of c-*erb*B-2 gene product is present in the cell lysate of SK-BR-3 breast cancer cells, which are known to express abundant c-*erb*B-2 mRNA (11); the three different antibodies against c-*erb*B-2 gene product, recognizing extracellular domain, kinase domain, and carboxyl-terminal portion, respectively, can specifically immunoprecipitate a large amount of ^{32}P-labeled and [^{35}S] cysteine-labeled protein with a molecular size of 185,000. The same protein was not immunoprecipitated when similarly treated A-431 cells were examined by these three antibodies. These findings suggest that the radiolabeled protein immunoprecipitated with these antibodies is the actual c-*erb*B-2 gene product present in SK-BR-3 cells and that this protein possesses all of these domains as well as the capacity to be phosphorylated. Radiolabeled c-*erb*B-2 gene product was further used for characterizing PoAbs and MoAbs developed in this study.

When mice were immunized with SK-BR-3 cells and then the antisera were used for immunoprecipitation, several phosphoproteins were immunoprecipitated from cell lysate of ^{32}P-labeled SK-BR-3 cells. The most intense band was regarded as an actual c-*erb*B-2 gene product for the following reasons: it appeared at the position identical to c-*erb*B-2 gene product with a molecular weight of 185,000; it possessed the activity to be phosphorylated; and the band was detected only in the cell lysate of SK-BR-3 cells. c-*erb*B-2 gene product present in the cell membrane probably stimulated the generation of these antibodies. Meanwhile, all mice immunized with concentrated SK-BR-3 cell culture medium unexpectedly developed antisera that immunoprecipitated the same radiolabeled-protein from the SK-BR-3 cell lysate. In this case, one major band was always observed, suggesting that the culture medium contained a small number of antigenic proteins with the capacity to be phosphorylated, when compared with whole cells. Antisera obtained from the mice immunized with concentrated culture medium conditioned by A-431 cells did not immunoprecipitate phosphorylated c-*erb*B-2 gene product; they immunoprecipitated phosphorylated EGFR with a M_r of 170,000. In contrast, antisera obtained from the mice immunized with fresh culture medium or FCS alone did not develop antibody against c-*erb*B-2 gene product or EGFR. These observations suggest that the culture medium conditioned by SK-BR-3 cells or A-431 cells contains a sufficient amount of substance(s) to induce antibodies against c-*erb*B-2 gene product and EGFR, respectively. Of interest is the fact that some of the PoAbs developed by injection of the SK-BR-3 cells as well as its culture medium can immunoprecipitate ^{32}P-labeled EGFR from cell lysate of A-431 cells. Since SK-BR-3 cells were known to possess a considerable amount of EGFR (12), it is reasonable to postulate that both EGFR on SK-BR-3 cells and EGFR-related protein in the culture medium induced the antibody against EGFR.

From the standpoint of biological sciences, successful development of an anti-c-*erb*B-2 gene product MoAb by immunizing mice with concentrated culture medium conditioned by SK-BR-3 cells indicates that this medium is a potent source of immunogen to develop MoAbs against c-*erb*B-2 gene product, a possible growth factor receptor. As described later, the newly developed MoAb, GFD-OA-p185-1, possesses similar recognition characteristics as the MoAb SV2-61γ, recognizing the extracellular domain of c-*erb*B-2 gene product. Therefore, we conclude that the culture medium conditioned by SK-BR-3 cells contains the substance(s) immunologically indistinguishable from c-*erb*B-2 gene product and desig-

nate this protein as c-*erb*B-2 gene product-related protein.

The biochemical nature of the c-*erb*B-2 gene product-related protein present in the culture medium conditioned by SK-BR-3 cells was further characterized. When the culture medium conditioned by [^{35}S]cysteine-labeled SK-BR-3 cells was immunoprecipitated by three different antibodies against c-*erb*B-2 gene product, no band with a molecular weight of 185,000 was observed; instead, the antibody (SV2-61γ) recognizing the extracellular domain precipitated a [^{35}S]cysteine-labeled protein with a molecular weight of 110,000, namely p110. The newly developed MoAb, GFD-OA-p185-1, also immunoprecipitated this protein, but the other two antibodies, recognizing kinase domain and carboxyl-terminal portion, did not. p110 is likely to be a specific product of SK-BR-3 cells, since this protein was not detected when the similarly treated culture medium conditioned by [^{35}S]cysteine-labeled A-431 cells was examined by antibodies for c-*erb*B-2 gene product. When the culture medium conditioned by ^{32}P-labeled SK-BR-3 cells was examined in the same manner, no labeled protein was immunoprecipitated by the four anti-c-*erb*B-2 gene product antibodies. Accordingly, it seems that p110 does not possess the capacity to be phosphorylated. These observations suggest that p110 is the actual c-*erb*B-2 gene product-related protein present in the culture medium conditioned by SK-BR-3 cells; also, p110 possesses the same or very similar structure to that of the extracellular domain of c-*erb*B-2 gene product. This situation would be comparable to the case of EGFR-related protein present in the culture medium conditioned by A-431 cells.

Considering the origin of p110, it seems that p110 is a specific product generated from c-*erb*B-2 gene product present on the cell membrane of SK-BR-3 cells. Alternatively, p110 is a molecule actively secreted from SK-BR-3 cells. Mayes and Waterfield (1) and Weber *et al.* (2) reported that the EGFR-related protein with a molecular size of 105,000 was present in the culture medium of A-431 cells; this protein has the activity to bind to epidermal growth factor and possesses the same amino acid sequence as the amino-terminal portion of EGFR. In the present study, EGFR-related protein with a molecular weight of 115,000 in our calculation was detected in the culture medium of A-431 cells; this medium when injected into mice generates an antiserum against EGFR. Since c-*erb*B-2 gene product and EGFR are similar proteins and the biochemical nature of p110 may be similar to EGFR-related protein, it is likely that p110 produced by SK-BR-3 cells is the counterpart of the EGFR-related protein, namely c-*erb*B-2 gene product-related protein. With the interleukin 4 receptor, a soluble form of this receptor was found with a distinct mRNA (13). In this context, it is worth noting that SK-BR-3 cells expressed two different c-*erb*B-2 mRNAs: one had a molecular size of 5.0 kb and was detected with five probes recognizing portions corresponding to extracellular, transmembrane, and cytoplasmic domains; the other was 2.8 kb and was detected only with three probes recognizing the portion corresponding to extracellular domain. These findings raise the possibility that the c-*erb*B-2 mRNA with the smaller molecular size encodes p110. Yamamoto *et al.* (5) demonstrated two c-*erb*B-2 mRNAs in a gastric cancer cell line, MKN-7 cells, with a molecular size similar to those detected in the present study. Accordingly, this heterogeneity of c-*erb*B-2 mRNA is not restricted to SK-BR-3 cells.

The above data support the concept that the c-*erb*B-2 gene product-related protein present in the SK-BR-3 culture medium is a soluble form of a c-*erb*B-2 gene product, a possible growth factor receptor. Further studies will be required to elucidate whether c-*erb*B-2 gene product-related protein is an actual product of c-*erb*B-2 gene.

Materials and Methods

Materials. BALB/c mice were provided by Japan Charles River Co., Ltd. (Atsugi, Kanagawa, Japan). $^{32}P_i$ (specific activity, 315–337 TBq/mmol) and [^{35}S]cysteine (specific activity, greater than 22.2 TBq/mmol) were purchased from New England Nuclear (Boston, MA).

Rabbit PoAb recognizing carboxyl-terminal portion of c-*erb*B-2 gene product, pAb 1 (T4881), was purchased from Triton Biosciences, Inc. (Alameda, CA); this was generated to a synthetic peptide from the carboxyl-terminal 14-amino acid sequence (1242–1255) of this protein (14), according to the putative amino acid sequence of c-*erb*B-2 gene product (5). Affinity-purified rabbit PoAb recognizing kinase domain of this protein, Ab-1, was purchased from Oncogene Science, Inc. (Manhasset, NY); a synthetic peptide composed of 15 amino acids with sequences of c-*erb*B-2 gene product (866–880) was used as the immunogen. Mouse MoAb (SV2-61γ) recognizing extracellular domain of c-*erb*B-2 protein was purchased from Nichirei Co. (Tokyo, Japan); this MoAb was developed by the method reported previously (15) and was characterized with the subtype of IgG1 according to the manufacturer's instruction. Anti-EGFR MoAb (RPN 513), recognizing an antigenic determinant located on the extracellular domain (16), was purchased from Amersham International Plc (Buckinghamshire, United Kingdom); this MoAb was generated with trypsinized A-431 cells as an immunogen and characterized to be IgG2a antibody. These antibodies were used according to the manufacturer's instructions.

Other materials purchased were: RPMI-1640 medium from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan); *p*-nitrophenylphosphate and polyacrylamide from Bio-Rad (Richmond, CA); BSA (Cohn Fraction V) from Dai-ichi Pure Chemicals (Osaka, Japan); Hepes and PMSF from Sigma Chemical Co. (St. Louis, MO); sodium pyrophosphate and SDS from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); sodium fluoride from Kanto Chemical Co. (Tokyo, Japan); protein A-Sepharose from Pharmacia (Uppsala, Sweden); hypoxanthine, aminopterin, and thymidine from Flow Laboratories (Irvine, Scotland); polyethylene glycol 4000 from Merck (Darmstadt, Federal Republic of Germany).

Cell Lines. A human breast adenocarcinoma cell line (SK-BR-3) was obtained from the American Type Culture Collection (Rockville, MD), and a human epidermoid carcinoma cell line (A-431) was provided from the Japanese Cancer Research Resources Bank (Tokyo, Japan). They were cultured in RPMI-1640 medium supplemented with 5% heat-inactivated FCS (Boehringer, Mannheim, Federal Republic of Germany), penicillin (100 units/ml), and streptomycin (200 mg/ml) in 225-cm^2 plastic tissue culture flasks (Sumilon; Sumitomo-Bakelite, Tokyo, Japan).

Myeloma cell line P3/X63-Ag8U1 (17) was kindly provided from the Pathology Division, National Cancer Center Research Institute (Tokyo, Japan); this cell line was cultured in RPMI-1640 medium supplemented with 10% heat-inactivated calf bovine serum (Hyclone Laboratories, Inc., Logan, UT) in 100-mm tissue culture dishes (Corning, NY) in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Northern Blot Analysis. Polyadenylated RNA extraction, gel electrophoresis, and Northern blot hybridization were performed by the previously described method (18). Five synthetic oligodeoxyribonucleotide probes designed to hybridize to the portions of c-*erb*B-2 mRNA corresponding to the extracellular, transmembrane, and cytoplasmic domains were used in the present study (Fig. 1). The structures of these probes were: Probe 1, 5′ CGC GGC TCC GGG GGG CAA GAG GGC GAG GAG GAG CCC CCA GCG GCA CAA GGC CGC CAG CTC 3′; Probe 2, 5′ GCC CTG GTA GAG GTG GCG GAG CAT GTC CAG GTG GGT CTC GGG ACT GGC AGG GAG CCG 3′; Probe 3, 5′ GGT GAC CAG GGC TGG GCA GTG CAG CTC ACA GAT GCC ACT GTG GTT GAA GTG GAG GCA GGC 3′; Probe 4, 5′ GAT GAG GAT CCC AAA GAC CAC CCC CAA GAC CAC GAC CAG CAG AAT GCC AAC CAC CGC AGA GAT GAT 3′; and Probe 5, 5′ TGG AGA CCT GGG GGC CTC CTC TTC AGA GGG CTC CAG CCC TAG TGT CAG GTC CCC ACC GCC 3′. With the aim of determining the integrity of cellular polyadenylated RNA extracted, the expression of human β-actin mRNA was examined by the previously described method (19). Ribosomal RNAs (28S and 18S) prepared from calf liver (Pharmacia) were used as molecular size markers.

^{32}P-labeled Proteins in Cell Lysate for Characterization of Antibodies. PoAbs and MoAbs developed in this study were characterized by ^{32}P-labeled proteins in SK-BR-3 and A-431 cells, according to the method reported previously (20). Cells in confluence of a density of 1×10^7 cells/100-mm dish were washed with phosphate-free Krebs-Ringer buffer, pH 7.4, containing 119 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 50 mM Hepes, and 0.1% (w/v) BSA, and then labeled with $^{32}P_i$ (18.5 MBq/ml) in 2 ml of the same buffer for 2 h at 37°C. The cells were solubilized with the lysis buffer, pH 7.4, containing 50 mM Hepes; 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA; 2 mM PMSF, 1% Triton X-100, and 400 μM sodium orthovanadate for 1 h at 4°C. Insoluble material was removed by centrifugation at 12,000 × *g* for 10 min. These cell lysates with ^{32}P-labeled proteins were used for immunoprecipitation, followed by SDS-PAGE with 7.5% polyacrylamide gel, in order to determine whether PoAbs recognize c-*erb*B-2 gene product or EGFR and to screen the antibody production by hybridomas.

Preparation of Culture Medium for Immunization. SK-BR-3 and A-431 cells were seeded into 225-cm^2 plastic tissue culture flasks each containing 50 ml of RPMI-1640 medium with 5% FCS. When these cells were grown to confluence, they were washed with serum-free medium, and then 50 ml of the same medium were added. After incubation for 72 h at 37°C, the medium was collected aseptically and freed from cell debris. A total of 200 ml of the serum-free medium obtained were concentrated 20-fold and 80-fold by ultrafiltration under nitrogen using a Diaflo cell type 8200 (Amicon Corp., Danvers, MA) fitted with a Diaflo YM-10 membrane (nominal M_r cutoff, 10,000; Amicon). The concentrates were sterilized with a 0.22-μm Millipore filter unit and stored at −80°C.

Preparation of PoAbs. Thirty BALB/c mice were divided into five groups and then immunized with the following substances three times weekly by i.p. injection: Group 1, 200 μl of FCS alone ($n = 3$); Group 2, 200 μl of 20-fold concentrated RPMI-1640 medium ($n = 5$); Group 3, 10^6 SK-BR-3 cells in 200 μl of serum-free RPMI-1640 medium ($n = 6$); Group 4, 200 μl of 20-fold concentrated culture medium conditioned by SK-BR-3 cells ($n = 11$); and Group 5, 200 μl of 20-fold concentrated culture medium conditioned by A-431 cells ($n = 5$). The fourth boost injection was done as follows: Group 1, 200 μl of FCS; Group 2, 200 μl of 80-fold concentrated RPMI-1640 medium; Group 3, 10^6 SK-BR-3 cells in 200 μl of serum-free RPMI-1640 medium; Group 4, 200 μl of 80-fold concentrated culture medium conditioned by SK-BR-3 cells; and Group 5, 200 μl of 80-fold concentrated culture medium conditioned by A-431 cells. Three days later, serum obtained from the mice was tested by immunoprecipitation for the presence of antibodies against c-*erb*B-2 gene product and EGFR.

MoAb Production. BALB/c mice were immunized i.p. with 20-fold concentrated culture medium conditioned by SK-BR-3 cells three times weekly. Seven days after the final i.p. injection, the mice were boosted with 200 μl of 80-fold concentrated cultured medium of SK-BR-3 cells. The spleen was removed 3 days later, and spleen cells were fused with nonsecreting P3/X63-Ag8U1 myeloma cells by the method reported previously (21) with a slight modification; for the fusion, 50% (w/v) polyethylene glycol 4000 was used.

Hybrid cells were grown in 96-well tissue culture dishes in RPMI-1640 containing 5 mM hypoxanthine, 20 μM aminopterin, and 800 μM of thymidine and supplemented with 10% calf bovine serum. Aminopterin was omitted from the medium after 2 weeks in culture. Hybridomas secreting antibodies reactive with membrane antigens of SK-BR-3 cells were identified by the screening method described below.

Screening Assays. The membrane fraction was isolated from SK-BR-3 cells. After culturing in RPMI-1640 medium containing 5% FCS, confluent SK-BR-3 cells were washed with phosphate buffer, pH 7.3, containing 10 mM phosphate and 15 mM NaCl, and then scraped. The cells collected by centrifugation at 400 × *g* at 4°C were homogenized (20 strokes) in hypotonic buffer, pH 7.4, containing 20 mM 1,4-piperazinediethane sulfonic acid-NaOH, 1 mM $MgCl_2$, and 5 mM KCl. The homogenate was first centrifuged at 1,500 × g for 5 min at 4°C, and then the supernatant was centrifuged at 100,000 × *g* for 30 min. The pellets were used as the membrane fraction to screen for positive clones by ELISA as described below.

Each well of 96-well microtiter plates was coated by incubation for 8 h at 4°C with crude membrane fractions prepared from 10^6 SK-BR-3 cells dissolved in 50 μl of Tris buffer, pH 7.5, containing 20 mM Tris base and 500 mM NaCl (TBS). The wells were washed three times with TBS containing 0.05% Tween 20 and then incubated with 300 μl/well of 0.1% (w/v) BSA in TBS for 1 h at 20°C to saturate nonspecific protein-binding sites. Subsequently, wells were washed three times with TBS containing 0.05% Tween 20. Hybridoma supernatant, 50 μl/well, was then added to the appropriate wells. After 1 h at

20°C, the wells were washed, and 50 μl of a 1:3,000 dilution of goat anti-mouse Immunoglobulin coupled to alkaline phosphatase (Bio-Rad) were added to each well. The plates were incubated for 1 h at 20°C and then washed to remove the unbound conjugate. Fifty μl/well of the enzyme substrate solution containing 5 mg of *p*-nitrophenylphosphate were added, and the color development was stopped by adding 50 μl/well of 0.5 M NaOH. Absorbance was measured with an Immuno Reader NJ2000 (Intermed, Tokyo, Japan). The positive clones were further screened by examining whether the clone possessed the activity to immunoprecipitate the phosphorylated c-*erb*B-2 gene product by the method described above.

Production and Purification of Antibody from Cloned Hybrids. One clone that secreted antibody recognizing c-*erb*B-2 gene product was the third limited dilution and was named GFD-OA-p185-1. In each limited dilution, clones were selected first by ELISA and then by immunoprecipitation of ^{32}P-labeled c-*erb*B-2 gene product in SK-BR-3 cell lysate. Culture medium of this clone was collected and further purified. Immunoglobulins were precipitated by addition of ammonium sulfate to 50% (w/v) saturation and redissolved in 140 mM sodium phosphate buffer, pH 8.0. Aliquots were applied to a Affi-gel Protein A-agarose column and were equilibrated in 140 mM sodium phosphate buffer, pH 8.0, at 4°C. After washing the column with the same buffer, bound antibody was eluted with 100 mM sodium citrate buffer, pH 3.0, according to the manufacturer's instruction. The eluate was pooled, adjusted to neutral pH, and stored at −20°C. The subclass of this MoAb was determined by ELISA based on isotype analysis (Bio-Rad).

^{32}P Labeling for Cell Lysate and Culture Medium. On the day before the cellular labeling, SK-BR-3 and A-431 cells were seeded at a density of 1×10^6 cells/35-mm dish. After washing the cells three times with phosphate-free RPMI-1640 medium without FCS, the cells were incubated with $^{32}P_i$ (18.5 MBq/ml) in 1 ml of phosphate-free Dulbecco's modified Eagle's medium (Nissui), pH 7.4, containing 5% FCS for 12, 24, and 48 h at 37°C. The cells were solubilized with 1 ml of the lysis buffer described in the previous section for 1 h at 4°C. Culture media (1 ml) were also collected. From the cell lysate and the culture media, insoluble material was removed by centrifugation at 12,000 × *g* for 10 min. Then, 200 μl of cell lysate or the culture media were used for immunoprecipitation.

[^{35}S]Cysteine Labeling for Cell Lysate and Culture Medium. On the day before the cellular labeling, SK-BR-3 and A-431 cells were seeded at a density of 3×10^6 cells/60-mm dish. Cells were washed with cysteine- and methionine-free RPMI-1640 medium without FCS, followed by preincubation with the same culture medium, and then they were incubated with [^{35}S]cysteine (3.7 MBq/ml) in 1.5 ml of cysteine-free RPMI-1640 medium with 1% FCS at 37°C for 20 and 48 h (SK-BR-3 cells) or 18 h (A-431 cells). The radiolabeled cells were solubilized with 1.5 ml of the lysis buffer described in the previous section for 1 h at 4°C. Culture media (1.5 ml) were also collected. From the cell lysate and the culture media, insoluble material was removed by centrifugation at 12,000 × *g* for 10 min. Then, 700 μl of cell lysate or the culture media were used for immunoprecipitation.

Immunoprecipitation and Electrophoresis. The cell lysate or the conditioned media from radiolabeled SK-BR-3 and A-431 cells was incubated with 10 μl of PoAbs, MoAbs, or NMS at 4°C for 3 h, and then Protein A-Sepharose (20 μl/sample) was added and further incubated at 4°C for 3 h. The solution was centrifuged at 12,000 × *g* for 10 min, and the precipitates were washed three times with the washing buffer, pH 7.4, containing 50 mM Hepes, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 4 mM EDTA, 0.05% Triton X-100, 400 μM sodium orthovanadate, and 2 mM PMSF. After the centrifugation, the precipitates were suspended in 50 μl of Laemmli's buffer, incubated at 110°C for 5 min, and then analyzed by SDS-PAGE, using 5.0 or 7.5% polyacrylamide gels (22). The following molecular markers were purchased from Bio-Rad: myosin (M_r 200,000), *Escherichia coli* β-galactosidase (M_r 116,250), phosphorylase *b* (M_r 97,400), BSA (M_r 66,200), and ovalbumin (M_r 42,699).

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