The Product of the Human c-*erb*B-2 Gene: A 185-Kilodalton Glycoprotein with Tyrosine Kinase Activity

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Antibodies were raised against a synthetic peptide corresponding to 14 amino acid residues at the COOH-terminus of a protein deduced from the human c-*erb*B-2 nucleotide sequence. These antibodies immunoprecipitated a 185-kilodalton glycoprotein from MKN-7 adenocarcinoma cells. Incubation of the immunoprecipitates with (γ-^{32}P)ATP resulted in the phosphorylation of this protein on tyrosine residues. These results indicate that the human c-*erb*B-2 gene product is the 185-kilodalton glycoprotein that is associated with tyrosine kinase activity. Although the c-*erb*B-2 protein was predicted to encode a protein very similar to epidermal growth factor (EGF) receptor, EGF did not stimulate this kinase activity either in vivo or in vitro.

THE V-*ERB*B GENE IS AN ONCOGENE of avian erythroblastosis virus, which induces erythroblastosis and sarcomas in susceptible chickens (*1, 2*). The v-*erb*B gene product possesses a sequence homologous to the kinase domain of transforming proteins of the *src* family (*3*) and has tyrosine kinase activity (*4*). Furthermore, the nucleotide sequence of the v-*erb*B gene has striking homology to that encoding the COOH half of the epidermal growth factor (EGF) receptor (*5*), a 170-kilodalton (kD) glycoprotein with tyrosine kinase activity (*6*), indicating that the v-*erb*B gene is derived from the chicken EGF receptor gene.

Recently, Semba *et al.* (*7*) have found a novel v-*erb*B–related gene, the c-*erb*B-2 gene, which is located on human chromosome 17 at q21 (*8*). The amino acid sequence predicted from the sequence of c-*erb*B-2 complementary DNA clones revealed that the gene encodes a possible receptor protein with close similarity to the EGF receptor (*9*). The c-*erb*B-2 gene is amplified in a human salivary adenocarcinoma (*8*), a gastric cancer cell line MKN-7 (*9*), and a mammary carcinoma (*10*), suggesting that elevated expression of the c-*erb*B-2 gene may be related to cellular transformation. In addition, comparison of the nucleotide sequences and deduced amino acid sequences of the c-*erb*B-2 gene and the *neu* gene—a v-*erb*B–related oncogene that was detected in a series of rat neuro/glioblastomas (*11*)—revealed that the *neu* gene is the rat counterpart of the c-*erb*B-2 gene (*9, 12*). In the present study, we have identified the c-*erb*B-2 gene product and characterized its enzymatic activity.

Antiserum to the c-*erb*B-2 gene product (anti–c-*erb*B-2) was prepared by immunizing rabbits with a synthetic peptide corresponding to amino acid residues 1242 to 1255 (Thr-Ala-Glu-Asn-Pro-Glu-Tyr-Leu-Gly-Leu-Asp-Val-Pro-Val) of the predicted protein. Specific antibodies were purified by affinity chromatography on a column to which the synthetic peptide had been covalently linked and were tested for the ability to immunoprecipitate the c-*erb*B-2 gene product from [^{35}S]methionine-labeled extracts of MKN-7 cells. The antibodies immunoprecipitated a 185-kD protein (Fig. 1A, lane 3) and precipitation was prevented by preincubating the antibodies with an excess amount of the synthetic peptide (Fig. 1A, lane 4). When the same experiment was performed with HeLa cells, in which only low levels of the c-*erb*B-2 transcripts were detectable by RNA blotting analysis (*13*), only small amounts of this 185-kD protein were detected (Fig. 1B, lane 3). By contrast, antibody to the EGF receptor (528 IgG) (*14*) immunoprecipitated the EGF receptor from HeLa cells (Fig. 1B, lane 2) as well as from MKN-7 cells (Fig. 1A, lane 2). These results suggest that the human c-*erb*B-2 gene product is the 185-kD protein that is similar in molecular weight to the *neu* gene product (*15*).

Since the predicted molecular weight of the primary product of the c-*erb*B-2 gene is 137,821 and the possible glycosylation sites are located at the extracellular domain (*9*), this 185-kD protein is expected to be a glycosylated form of the primary product. In fact, [^{3}H]glucosamine and [^{3}H]mannose are incorporated into the 185-kD protein by metabolic labeling (*13*). When anti–c-*erb*B-2 was reacted with a lysate of MKN-7 cells that had been treated with tunicamycin (2 μg/ml), which suppresses *N*-linked glycosylation (*16*), a 160-kD protein (but not a 135- to 140-kD protein) was specifically immunoprecipitated (Fig. 1A, lanes 7 and 8). This

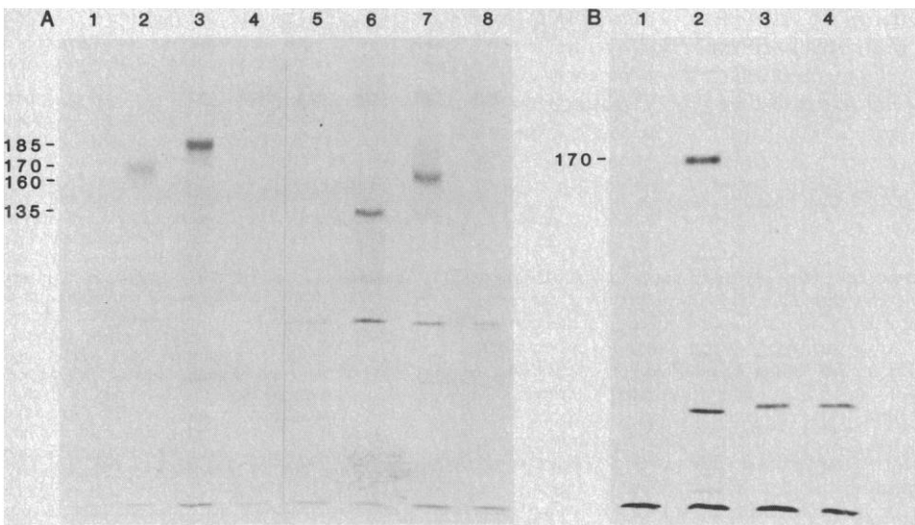

Fig. 1. Immunoprecipitation of the c-*erb*B-2 gene product. (A) MKN-7 cells were labeled for 6 hours (lanes 1 to 4) in Dulbecco's modified Eagle's medium (MEM) supplemented with 5% dialyzed fetal calf serum (FCS) and [^{35}S]methionine (100 μCi/ml, 1200 Ci/mmole, Amersham). (Lanes 5 to 8) MKN-7 cells were treated with tunicamycin (2 μg/ml) for the final 2 hours of [^{35}S]methionine-labeling. The cells were lysed in RIPA buffer [40 m*M* Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-NaOH, *p*H 7.4, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 0.15*M* NaCl, 1 m*M* phenylmethylsulfonyl fluoride (PMSF)]. Aliquots (150 μl) of the lysates were incubated with normal rabbit serum (lanes 1 and 5), antibody to the EGF receptor (528 IgG) that reacts with the extracellular domain of the receptor (*14*) (lanes 2 and 6), anti–c-*erb*B-2 prepared as described (*20*) (lanes 3 and 7), or anti–c-*erb*B-2 that had been preabsorbed with an excess amount of the synthetic peptide used for immunization (lanes 4 and 8) for 1 hour on ice. (B) HeLa cells, labeled and treated as in panel A, lanes 1 to 4, respectively. The immunocomplexes were adsorbed to protein A–Sepharose 4B (Pharmacia) and washed extensively with RIPA buffer (*20*). The immunoprecipitates were analyzed on an SDS–polyacrylamide (7.5%) gel followed by fluorography as described (*21*). Sizes are shown in kilodaltons.

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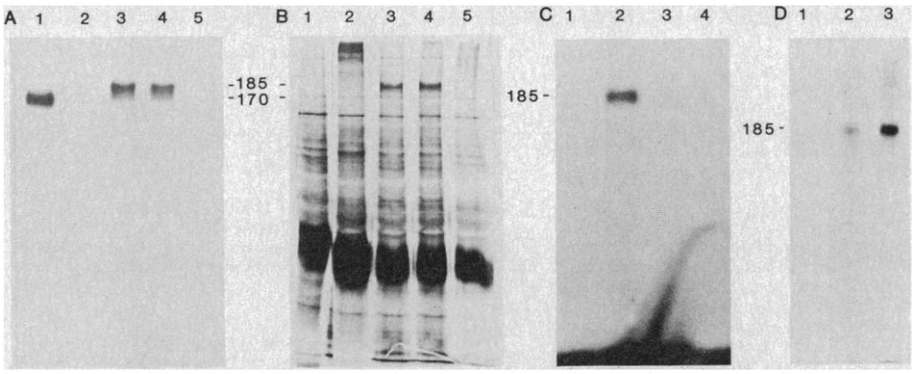

Fig. 2. Phosphorylation of the c-*erb*B-2 gene product in vitro and in vivo. (A and B) MKN-7 cells were lysed in solubilizing buffer (40 m*M* Hepes-NaOH, *p*H 7.4, 1% Triton X-100, 10% glycerol, 1 m*M* PMSF) and then immunoprecipitated with antibody to the EGF receptor (528 IgG) (lanes 1), normal rabbit serum (lanes 2), anti–c-*erb*B-2 (lanes 3 and 4), or anti–c-*erb*B-2 that had been preabsorbed with an excess amount of the synthetic peptide (lanes 5). The immunoprecipitates were incubated with (γ-^{32}P)ATP (20 μM, 4 mCi/mmol) in a final volume of 60 μl containing 40 m*M* Hepes-NaOH, *p*H 7.4, 10 m*M* $MgCl_2$, 3 m*M* $MnCl_2$, and 0.05% Triton X-100. For lanes 4, EGF (1 μg/ml) was included in the reaction mixture. After 5 minutes at 25°C, the reactions were terminated by the addition of Laemmli's SDS sample buffer and boiled for 2 minutes. The samples were subjected to SDS–polyacrylamide gel electrophoresis, stained with silver (B), and analyzed by autoradiography(A). (C) HeLa cells, lysed as described above, were immunoprecipitated with normal rabbit serum (lane 1), antibody to the EGF receptor (lane 2), anti–c-*erb*B-2 (lane 3), or anti–c-*erb*B-2 that had been preabsorbed with an excess of synthetic peptide (lane 4). (D) MKN7 cells were labeled for 7 hours in phosphate-free Dulbecco's MEM supplemented with 2% dialyzed FCS and ^{32}P-orthophosphate (300 μCi/ml, Amersham). EGF (50 ng/ml) was added for the last 15 minutes (lane 3). The labeled cells were lysed, reacted with anti–c-*erb*B-2 (lanes 2 and 3) or anti–c-*erb*B-2 that had been preabsorbed with an excess of the synthetic peptide (lane 1), and analyzed as in Fig. 1.

cannot be explained by incomplete inhibition of glycosylation because a protein of the same size was precipitated from MKN-7 cells treated with a higher concentration of tunicamycin (30 μg/ml). In the presence of tunicamycin, the EGF receptor was observed as a precursor form of 140 kD (Fig. 1A, lane 6) (*17*). Therefore, the c-*erb*B-2 gene product may be modified post-translationally by *O*-linked glycosylation as well as by *N*-linked glycosylation.

An intracellular domain of the c-*erb*B-2 gene product contains a stretch of 260 amino acids (residues 727 to 986) that is highly homologous to the conserved region present in all tyrosine kinases (*9*). In addition, the tyrosine residues at positions 1139, 1222, and 1248 of the c-*erb*B-2 gene product are at equivalent positions to those autophosphorylated in the EGF receptor (*18*). We, therefore, examined the autophosphorylation activity of the c-*erb*B-2 gene product in vitro. Immunoprecipitates prepared from detergent lysates of MKN-7 cells were incubated with (γ-^{32}P)ATP and analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. Anti–c-*erb*B-2 reacted with one major phosphoprotein (185 kD; Fig. 2A, lane 3) that was distinct from the autophosphorylated EGF receptor (Fig. 2A, lane 1) and was not immunoprecipitated by anti–c-*erb*B-2 that had been preabsorbed with an excess of the synthetic peptide (Fig. 2A, lane 5). Addition of EGF to the kinase reaction did not enhance the phosphorylation of the 185-kD protein (Fig. 2A, lane 4). In an immune-complex kinase assay with HeLa cell lysates, a 185-kD phosphoprotein was not detected with anti–c-*erb*B-2, while the phosphorylated EGF receptor was readily detected with antibodies to the EGF receptor (Fig. 2C). These results suggest that the 185-kD phosphoprotein is the autophosphorylated c-*erb*B-2 gene product.

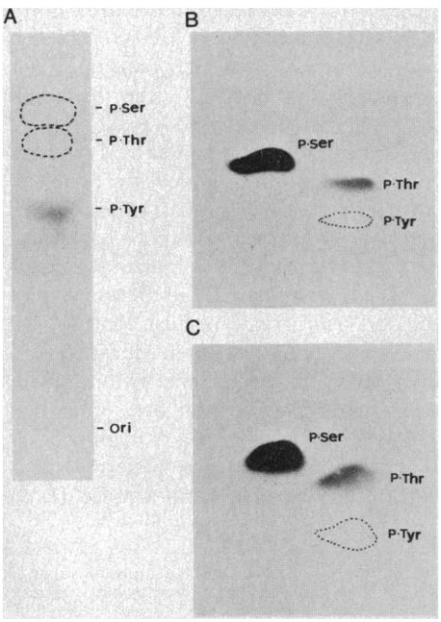

Fig. 3. Phosphoamino acid analysis of the c-*erb*B-2 gene product. The c-*erb*B-2 gene product phosphorylated in vitro (A), in vivo (B), or in vivo in the presence of EGF (50 ng/ml) (C) was immunoprecipitated and analyzed as described in Fig. 2. The phosphorylated 185-kD protein was eluted from the gel and subjected to acid hydrolysis in 6*N* HCl for 1.5 hours at 110°C. The phosphoamino acids were resolved by electrophoresis at *p*H 3.5 (A) or by two-dimensional electrophoresis at *p*H 1.9 and *p*H 3.5 (B and C) on cellulose thin-layer plates (*22*).

Phosphorylation of the 185-kD protein occurred exclusively on tyrosine residues (Fig. 3A). The 185-kD protein was present in higher amounts but showed less autophosphorylation activity than the EGF receptor (Fig. 2, A and B). This may result from the blocking of the autophosphorylation site by antibodies to c-*erb*B-2, which are directed against the region including the possible autophosphorylation site at 1248.

The c-*erb*B-2 gene product was also identified as a phosphorylated 185-kD protein after labeling in vivo (Fig. 2D). Phosphoamino acid analysis, however, showed that the c-*erb*B-2 gene product was mainly phosphorylated on serine and threonine residues (Fig. 3B). When EGF was added to MKN-7 cells during the last 15 minutes of ^{32}P-labeling, phosphorylation of the c-*erb*B-2 gene product was markedly increased (Fig. 2D, lane 3). This enhanced phosphorylation was due to the increase of phosphoserine and phosphothreonine but not to the appearance of phosphotyrosine (Fig. 3C). This is consistent with the observation that the tyrosine kinase activity of the c-*erb*B-2 gene product in vitro is not enhanced by the addition of EGF (Fig. 2A). Furthermore, the c-*erb*B-2 gene product does not bind to EGF (*13*). Thus, phosphorylation of the c-*erb*B-2 gene product may be indirectly stimulated by EGF through an as yet unidentified pathway involving the EGF receptor.

The c-*erb*B-2 gene product was shown to be a 185-kD glycoprotein that is associated with tyrosine kinase activity. Similar kinase activities are associated with several cellular growth-factor receptors as well as with transforming proteins of the *src* family (*19*), and are assumed to be involved in the regulation of cell growth and cell transformation. Since the c-*erb*B-2 gene product consists of the extracellular ligand-binding domain and the intracellular kinase domain (*9*), it is possible that binding of growth factor to the c-*erb*B-2 gene product stimulates its tyrosine kinase activity and may be important in transmission of mitogenic signals.

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Different Mutations in Ashkenazi Jewish and Non-Jewish French Canadians with Tay-Sachs Disease

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Tay-Sachs disease patients of Ashkenazi Jewish and non-Jewish French Canadian origin are affected with a clinically identical form of this inherited disease. Both have a similar gene frequency for the disorder, which is tenfold higher than that found in the general population. Unlike other patients with the disease, who often display variation at the clinical or biochemical level, the absence of such differences between these two groups has prompted the idea that they may harbor the same mutation. In this report, a complementary DNA clone coding for the α chain of human β-hexosaminidase has been used to analyze the genetic lesions in the α-chain locus of two patients with Tay-Sachs disease from each of these groups. On the basis of DNA hybridization analyses, the α-chain gene of the Ashkenazi patients appears intact while the α-chain gene of French Canadian patients has a 5′ deletion of approximately 5 to 8 kilobases.

TAY-SACHS DISEASE IS AN INHERITED disorder caused by mutation in the α chain of β-hexosaminidase A, a lysosomal enzyme composed of two polypeptides designated the α and β chains (*1*, *2*). Deficiency of β-hexosaminidase A results in storage of its major substrate, G_{M2} ganglioside. Progressive accumulation of substrate leads to the characteristic neurodegenerative changes in patients with Tay-Sachs disease. The disease is heterogeneous (*1*–*4*), displaying a wide range of severity and age of onset. An early onset and fatal form of the disorder referred to as "classic" Tay-Sachs disease has a tenfold higher gene frequency among Ashkenazi Jews than the general population. A less publicized group having a carrier frequency equal to that of Ashkenazi Jews is a population of non-Jewish French Canadians located in eastern Quebec (*5*). In terms of age-of-onset, clinical course, and biochemical parameters (*6*), French Canadian patients are indistinguishable from Ashkenazi patients. Identity of mutation has been proposed as one possible explanation for the similarities between these two groups at risk for classic Tay-Sachs disease (*5*). We have recently observed that polyadenylated [poly(A)$^+$] RNA preparations from five Ashkenazi (*7*) and two non-Jewish French Canadian (*8*) Tay-Sachs patients lacked detectable α-chain message when analyzed by Northern blotting, with complementary DNA (cDNA) encoding the α chain of human β-hexosaminidase. By means of a cDNA coding for the entire α-chain polypeptide (*9*), we have now analyzed and compared the α chains of Ashkenazi Jewish and French Canadian patients at the DNA level.

DNA was isolated from cultured fibroblasts of two non-Jewish French Canadian patients and two Ashkenazi patients. DNA for controls was obtained either from cultured cells or from blood leukocytes of normal subjects. Each sample was digested with Eco RI and analyzed by Southern blotting with a ^{32}P-labeled cDNA probe (pβHα-5) containing the entire coding sequence for the α chain of human β-hexosaminidase (*9*). An identical restriction pattern was obtained for the normal controls and Ashkenazi Tay-Sachs samples; these consisted of three DNA fragments with approximate sizes of 6, 4, and 3 kilobases (kb) (Fig. 1). However, the 4-kb DNA fragment was absent from the restriction pattern of both French Canadian Tay-Sachs samples. (A re-

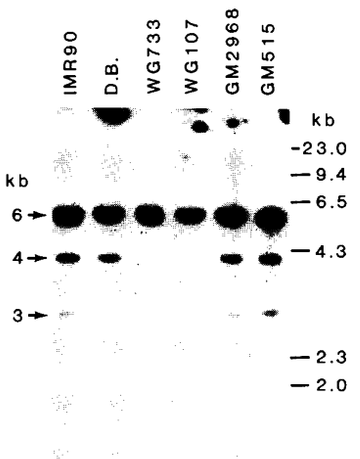

Fig. 1. Southern blot analysis of DNA from normal individuals and from patients with Tay-Sachs disease of French Canadian and Ashkenazi origin. Normal (IMR90) and Ashkenazi Tay-Sachs (GM515, GM2968) fibroblast cultures were obtained from the Human Genetic Cell Repository, Institute for Medical Research, Camden, NJ. The cells from non-Jewish French Canadian Tay-Sachs patients (WG107, WG733) were obtained from the Repository for Human Mutant Cell Strains, Montreal, Canada. DNA was isolated from these cultures (*11*) as well as from whole blood leukocytes (*12*) of a normal non-Canadian Ashkenazi individual (D.B.) who was not a heterozygote carrier for Tay-Sachs disease. Genomic DNA of normals and mutants (7 μg) was digested to completion with Eco RI, fractionated on a 1% agarose gel and transferred to GeneScreen Plus (New England Nuclear) in 10× standard saline citrate. Transferred DNA was then hybridized at 42°C in the presence of formamide (*11*) to the insert of pβHα-5, a cDNA clone that contains the entire coding sequence for the α chain of human β-hexosaminidase (*9*) and that had been labeled with ^{32}P to a specific activity of 2×10^9 count/min per microgram of DNA by the random primer method (*13*). Blots were washed as suggested by the manufacturer and exposed for 36 hours to x-ray film by means of a Cronex Hi Plus intensifying screen.

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