J. Biochem. 122, 675-680 (1997)

A Novel Brain-Derived Member of the Epidermal Growth Factor Family That Interacts with ErbB3 and ErbB4[1]

Shigeki Higashiyama,*,[2] Michiharu Horikawa,* Kouji Yamada,† Naohiro Ichino,† Norihiko Nakano,* Takatoshi Nakagawa,* Junichiro Miyagawa,‡ Natsuki Matsushita,† Toshiharu Nagatsu,† Naoyuki Taniguchi,* and Hiroshi Ishiguro†,[2]

*Departments of *Biochemistry and ‡Internal Medicine, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565; and the †Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-11*

Received for publication, June 27, 1997

A novel member of the epidermal growth factor (EGF) family, the neural- and thymus-derived activator for ErbB kinases (NTAK), has been purified and cloned. Five alternative spliced isoforms have been detected in the rat adrenal pheochromocytoma cell line, PC-12 cells. The rat NTAKα2a isoform exhibits 94% identity in its primary sequence with the human NTAKα isoform. *In vivo*, NTAK is only expressed in the brain of rat E11.5 embryos, and in the brain and thymus of adult rats. The soluble 46 kDa form binds directly to ErbB3 and B4, but not to ErbB1 or B2. NTAK, however, transactivates ErbB1 and B2 *via* heterodimerization with ErbB3 or B4. NTAK stimulates the differentiation of MDA-MB-453 cells and competitively inhibits the binding of [^{125}I]neuregulin to these cells. In addition to these neuregulin-like properties, NTAK exhibits limited structural homology to neuregulins in the immunoglobulin (Ig)-like, EGF-like, and hydrophobic domains. Thus, NTAK appears to be a new member of the EGF family displaying neuregulin properties.

Key words: EGF, ErbB, neuregulin, NTAK, PC-12.

The ErbB family, which are membrane protein tyrosine kinases, encompass four members, ErbB1 (EGF receptor), B2 (neu), B3, and B4 (*1-4*). ErbB ligand-receptor interactions have been shown to play crucial roles in embryonic development (*5-10*) and are associated with certain human malignancies (*11*). Studies on *ErbB* knockout mice have clearly demonstrated that ErbB1 is essential for epithelial development in the skin, lung, and gastrointestinal tract (*5-7*). In addition, knockout studies have shown that ErbB2 and B4 are crucial for the development of the cardiac muscle and central nervous systems (*9, 10*).

Direct ligands for these receptors, except for ErbB2, have been identified. EGF (*12*), transforming growth factor-α (*13*), amphiregulin (*14*), heparin-binding EGF-like growth factor (HB-EGF) (*15*), betacellulin (*16*), and epiregulin (*17*) have been identified as ligands for ErbB1. On the other hand, neuregulins are known to bind directly to ErbB3 and B4, and to transactivate ErbB1 and B2 *via* heterodimerization with ErbB3 and B4 (*18, 19*).

In order to investigate other possible ErbB3 and/or B4 ligands, RT-PCR was carried out to clone PC-12-derived ErbB3 and/or B4 ligands using primers sandwiching the EGF-like domain of neuregulin (*20*), and then we cloned a gene encoding a novel direct ligand, NTAK, for ErbB3 and B4. We also cloned a gene encoding the human homologue of NTAK.

EXPERIMENTAL PROCEDURES

PCR and cDNA Cloning of NTAK—cDNA was synthesized from poly(A)$^+$ RNA of PC12 cells by random hexamer priming using MMLV reverse transcriptase (Gibco). The following oligonucleotide primers were prepared: 5′-primer, 5′-AAGGAGAAAACTTTCTGTGTGAATGG-3′ (K^{185}EKTFCVNG of NDF, sense); and 3′-primer, 5′-GCAGTAGGCCACCACACACATGATGCC-3′ (G^{257}IMCVVAYC of NDF, antisense). PCR (*21*) was performed for 35 cycles comprising 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min. A Uni-ZAP XR cDNA library was prepared from poly(A)$^+$ RNA of PC-12 cells using a ZAP cDNA library kit (Stratagene) according to the instruction manual. Three hybridizing clones were purified and analyzed with an automatic DNA sequencer (Perkin-Elmer 373A). To analyze the isoforms of *NTAK*, the following PCR primers perfectly matching *NTAK* were synthesized: 5′-primer, 5′-ACAGCCAAGTCCTACTGTGTGAATGG-3′ (T^{364}AKSYCVNG, sense), and 3′-primer, 5′-ACAGTAGGCGACCACACAGACGATGCC-3′ (G^{427}IVCVVAYC, antisense). Human NTAK cDNA was cloned from the human neuroblastoma cell line, SK-NSH cells, using rat NTAK cDNA as a probe.

[1] The nucleotide sequence data for NTAK reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the following accession numbers D89995 (α1), D89996 (α2a), D89997 (β), D89998 (γ), and AB001576 (α2b) for rat, and AB005060 for human. This work was supported by grants to S. Higashiyama, N. Taniguchi, T. Nagatsu, and H. Ishiguro from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed: Shigeki Higashiyama, Tel: +81-6-879-3421, Fax: +81-6-879-3429, E-mail: shigeki@biochem.med.osaka-u.ac.jp; Hiroshi Ishiguro, Tel: +81-562-93-9393, Fax: +81-562-93-8831, E-mail: hishi@fujita-hu.ac.jp

Abbreviations: NTAK, neural- and thymus-derived activator for ErbB kinases; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; Ig, immunoglobulin; PCR, polymerase chain reaction.

Purification and NH_2-Terminal Sequence Analysis of Processed rNTAKα2a—Recombinant NTAKα2a was purified from NS-67 cell-conditioned medium (20 liters) by heparin affinity, Cu-chelate, and C_4-reversed phase column chromatographies based on HB-EGF purification procedures (*22*). The activity of NTAK was estimated as quantitating the increase in tyrosine phosphorylation of ErbB3 in MDA-MB-453 cells. The purified rNTAKα2a protein (8 μg) was separated by SDS-polyacrylamide gel electrophoresis, electroblotted onto a PVDF membrane, and then stained with Coomassie Brilliant Blue. A piece of membrane was directly applied to a Hewlett Packard protein sequencing system, HP G1005A.

Cell Lines and Transfectants—The NS-67 cell line was established from CHO-K1 cells by transfection with NTAKα2a cDNA subcloned into the pRc/CMV plasmid (Invitrogen). Mouse myeloid 32D cells (ATCC) were transfected transiently with human ErbB1, B2, B3, and B4 cDNAs subcloned into the pRc/CMV plasmid (20 μg each) singly or in pairwise combinations by electroporation (500 μF, 350 V). Human ErbB1 cDNA was a gift from Dr. M. Shibuya (The University of Tokyo), B2 cDNA from Dr. T. Akiyama (Osaka University), and B3 and B4 cDNAs from Dr. T. Arakawa (Amgen).

Surface Plasmon Resonance Analysis—The purified rNTAKα2a was immobilized on a CM5 sensor chip (Pharmacia Biosensor). The ectodomain purified from each ErbB (20 μl, 100 μg/ml in 10 mM phosphate-buffered saline) was injected at the flow rate of 5 μl/min. The ErbB1 ectodomain was purified from A-431 cell-conditioned medium by affinity chromatography on an anti-ErbB1 antibody column. The purified ectodomains of ErbB2, B3, and B4 were generous gifts from Dr. T. Arakawa. Surface plasmon resonance analyses were performed at 25°C with a BIAcore 2000 apparatus (Pharmacia Biosensor).

Immunostaining—An anti-NTAK rabbit antibody was raised against a synthetic peptide corresponding to the 308-327 amino acid sequence in Fig. 1. Western blot analyses revealed that the antibody does not crossreact with neuregulins (Higashiyama *et al.*, unpublished observation). Brain tissues of 4-week-old rats were perfusion-fixed with 4% paraformaldehyde, and then frozen sections were used for immunohistostaining of the NTAK protein by the avidin-biotin complex method (Vector Laboratories) using the rabbit anti-NTAK antibody. An excess amount of the peptide blocked NTAK immunostaining.

In Situ Hybridization—Whole-mount *in situ* hybridization was performed with an antisense *NTAK* probe labeled with digoxigenin. The mRNA was detected with alkaline phosphatase-conjugated anti-digoxigenin antibodies and 4-nitrotetrazolium chloride (Boehringer Mannheim RNA labeling and detection kit).

Northern Blotting Analysis of mRNA—Poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose affinity column chromatography (*23*). Poly(A)$^+$ RNA was electrophoresed in a 1% agarose gel, transferred to a HybondN$^+$ membrane (Amersham) by capillary diffusion, and then hybridized with a rat NTAK or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. The hybridized membranes were put into contact with a BAS1000 plate (Type BAS-IIIs), and then analyzed with a BAS1000 (Fuji Photo Film, Tokyo).

```
↓
MRQVCCSALP PP.LEKARCS SYSYSDSSSS SSSNNSSSST SSR.SSSRSS SRSSRGSTTT TSSSENSGSN SGSIFRPAAP PEPRPQPQPQ PRSPAARRAA   98
---------- --P---G--- ---D-S---- ER-SS----S -ESG------ -N..... .........- -.--S----- ------Q--- ----------   82

          ↓          ↓                                        *
ARSRAAAAGG MRRDPAPGSS MLLFGVSLAC YSPSLKSVQD QAYKAPVVVE GKVQGLAPAG GSSSNSTREP PASGRVALVK VLDKWPLRSG GLQREQVISV  198
---------- --------F- ---------- ---------- ---------- ------V--- ---------- ---------- ---------- ----------  182
                  α2b FFF┐
GSCAPLERNQ RYIFFLEPTE QPLVFKTAFA PVDPNGKNIK KEVGKILCTD CATRPKLKKM KSQTGEVGEK QSLKCEAAAG NPQPSYRWFK DGKELNRSRD  298
---V------ ---------- ---------- -L-T----L- ---------- ---------- -----Q---- ---------- ---------- ----------  282

Ig-like domain                                                                    EGF-like domain
IRIKYGNGRK NSRLQFNKVK VEDAGEYVCE AENILGKDTV RGRLHVNSVS TTLSSWSGHA RKCNETAKSY CVNGGVCYYI EGINQLSCKC PNGFFGQRCL  398
---------- ---------- ---------- ---------- ----Y----- ---------- ---------- ---------- ---------- ----------  382

                                hydrophobic domain
                                                                                *
EKLPLRLYMP DPKQKAEELY QKRVLTITGI CVALLVVGIV CVVAYCKTKK QRRQMHHHLR QNMCPAHQNR SLANGPSHPR LDPEEIQMAD YISKNVPATD  498
---------- ---------- ---------- ---------- ---------- --K---N--- ---------- ---------- ---------- ----------  482

HVIRREAETT FSGSHSCSPS HHCSTATPTS SHRHESHTWS LERSESLTSD SQSGIMLSSV GTSKCNSPAC VEARARRAAA YSQEERRRAA MPPYHDSIDS  598
------T--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- -NL------T A------V--  582

LRDSPHSERY VSALTTPARL SPVDFHYSLA TQVPTFEITS PNSAHAVSLP PAAPISYRLA EQQPLLRHPA PPGPGPGPGA D......MQR SYDSYYYPAA  692
---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------P GPGPGAD--- ----------  682

GPGPRRGACA LGGSLGSLPA SPFRIPEDDE YETTQECAPP PPPRPRTRGA SRRTSAGPRR WRRSRLNGLA AQRARAARDS LSLSSGSGCG SASASDDDAD  792
-------T-- ---------- ---------- ---------- ------A--- ---------- ---------- ---------- --------G- ----------  782

DADGALAAES TPFLGLRAAH DALRSDSPPL CPAADSRTYY SLDSHSTRAS SRHSRGPPTR AKQDSGPL   860  rat NTAKα2a
---------- -------G-- ---------- ---------- ---------- ---------P- -----A--   850  human NTAKα
```

Fig. 1. **NTAK structure.** Predicted primary translation products of rat NTAKα2a and human NTAKα. Underline, NH_2-terminal amino acid sequence of purified rr·NTAKα2a; asterisks, possible sites of *N*-linked glycosylation; arrows, possible translation initiation sites; hyphens, identical amino acids in rat and human. Gaps were inserted in the sequence for the best alignment. The NH_2-terminal amino acid of the rat NTAKα2b isoform has not been determined yet.

RESULTS AND DISCUSSION

Four of the cloned cDNAs were found to encode novel closely related sequences. Using the mixture of the cDNA fragments as a probe, three hybridizing clones were isolated from a cDNA library constructed from rat PC-12 cells. One of these clones encodes an open reading frame consisting of 2,580 bp. An 860 amino acid residue primary translation product of rat NTAK was predicted from this clone (Fig. 1). Human NTAK cDNA was also cloned from human SK-NSH cells. An 850 amino acid residue primary translation product was predicted from this clone (Fig. 1). Rat NTAKα2a and human NTAKα show 94% identity in their amino acid sequences. Amino acid hydropathy analysis (Fig. 2a) of rat NTAKα2a revealed a hydrophobic 23 amino acid stretch from valine 422 to cysteine 444. A typical signal peptide was not found at the NH_2-terminus. NTAK has three characteristic Ig-like, EGF-like, and hydrophobic domains, of which the amino acid sequences are completely identical in rat and human (Figs. 1 and 2a). Furthermore, an alternative splicing site in the EGF-like domain gives rise to at least four NTAK isoforms (α1, α2, β, and γ), and at phenylalanine 223 two NTAKα2 isoforms (α2a and α2b) (Figs. 1 and 2c). The predicted sequence of NTAK is novel and exhibits significant homology with neuregulin in the three characteristic domains. Between the first and sixth cysteines in the EGF-like domain, NTAKα and β are structurally most similar to rat neuregulin (49%) (*20*). There is 43-46% identity to rat EGF (*24*) (Fig. 2c). The hydrophobic domain of NTAK is 91% identical and the Ig-like domain 38% identical to the corresponding domains of neuregulin, respectively. However, the NH_2-terminal region upstream of the Ig-like domain and the cytoplasmic domain exhibit no structural homology with any member of the EGF family including neuregulins. Recently, neuregulin 2 was identified and revealed to be identical to NTAK (*25, 26*).

To characterize the biological activities of NTAK, rat recombinant NTAKα2a (rr•NTAKα2a) was purified from the conditioned medium of NS-67 cells, a stable clone of CHO-K1 cells transfected with full-length rat•NTAKα2a cDNA. The purified rr•NTAKα2a gave a 46 kDa diffuse band (Fig. 2b). Approximately 8 μg of the purified rr•NTAKα2a protein was subjected to microsequencing, yielding the unique NH_2-terminal amino acid sequence, X-Y-S-P-S-L-K-S-V-Q-D-Q-A-Y-K-A-P-V-V-V-E-G-K-V-Q-G-L-A-P-A-G-G-S-S-S, which matches amino acids 128 to 162 of the predicted primary sequence (Fig. 1), suggesting that methionine 109 might be a possible translation initiation site.

Since NTAK is structurally a new member of the EGF family, NTAK-ErbB interactions were examined in a purified protein-protein interaction system by means of surface plasmon resonance analyses. NTAK bound directly to the purified ectodomains of ErbB3 and B4, but not to that of ErbB1 or B2 (Fig. 3a). To investigate the biochemical effects of NTAK-ErbB interactions, the ability of rr•NTAKα2a to induce the tyrosine phosphorylation of ErbBs was examined using mouse myeloid 32D cell lines, which do not express any endogenous ErbB (*18*), but which were transfected with ErbB cDNA singly and in pairwise combi-

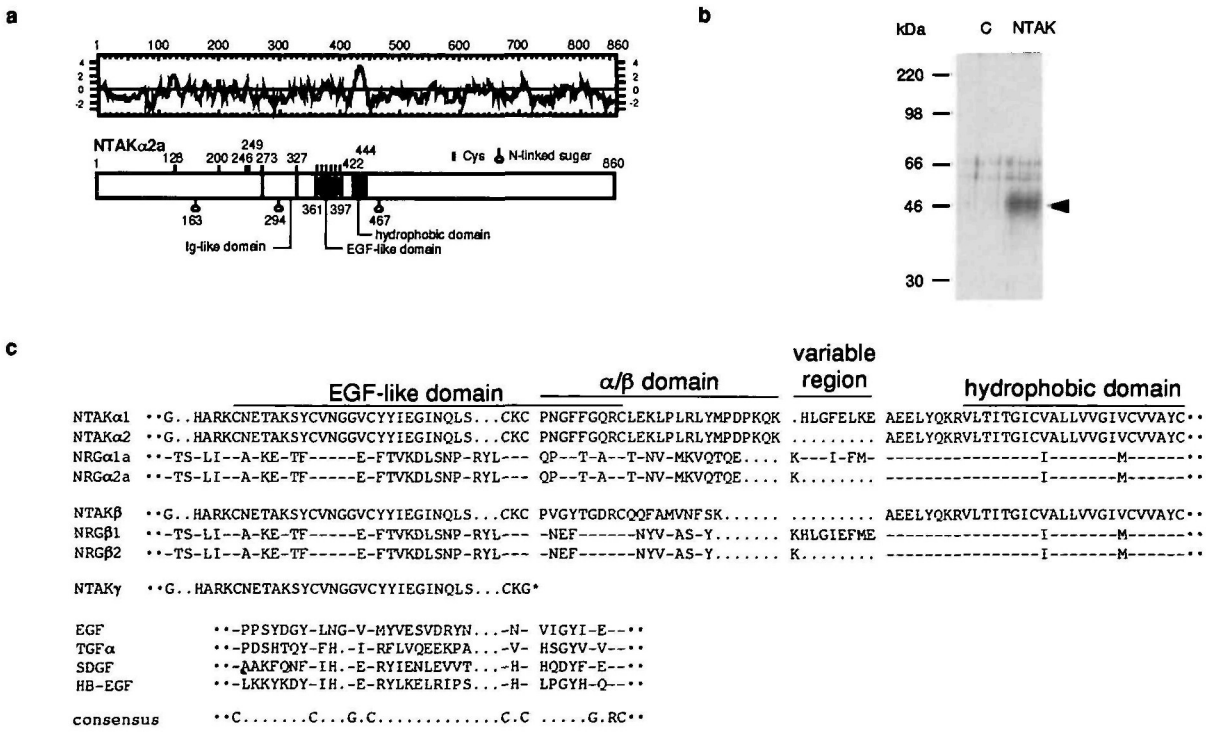

Fig. 2. **Rat NTAK protein purification and isoform analyses.** a: Scheme of the rat NTAKα2a primary translation product and hydropathy profile. b: SDS-polyacrylamide gel electrophoresis. Protein was visualized by silver staining (arrow head). Lane C, without sample; lane NTAK, approximately 100 ng of purified rr•NTAKα2a. c: Isoform analyses of rat NTAK and its alignment. The four isoforms of rat NTAK are aligned with rat neuregulin (NRG) isoforms (*27*) and other rat EGF family members (*28-30*). Hyphens, identical amino acids; asterisk, COOH-terminus. Gaps were inserted in the sequence for the best alignment.

nations. rr·NTAKα2a induced the tyrosine phosphorylation in 32D-B4 cells expressing ErbB4 alone, but not in 32D-B3 cells expressing ErbB3 alone, since these cells have impaired kinase activity. rr·NTAKα2a also did not stimulate the tyrosine phosphorylation in 32D-B1 or 32D-B2 cells (Fig. 3b). rr·NTAKα2a, however, significantly increased the tyrosine phosphorylation of both of the paired ErbB receptors in 32D cells expressing ErbB1/B3, B2/B3, B3/B4, B1/B4, and B2/B4 in pairwise combinations (Fig. 3b), suggesting that rr·NTAKα2a can transactivate ErbB1 and B2 *via* heterodimerization with ErbB3 or B4.

Since the ErbB cross-activation induced by rr·NTAKα2a was similar to that of neuregulin (*18, 19*), other activities of neuregulin were examined. MDA-MB-453 cells treated with rr·NTAKα2a or recombinant human neuregulinβ1 (rh·neuregulinβ1) were more adherent than controls. The treated MDA-MB-453 cells displayed a flat morphology with larger nuclei and increased cytoplasm, whereas the untreated cells were round and only moderately adherent (Fig. 4a). Purified rr·NTAKα2a induced ErbB2 and B3 tyrosine phosphorylation in MDA-MB-453 cells as did rh·neuregulinβ1 (Fig. 4b). Furthermore, rr·NTAKα2a was found to inhibit by more than 90% the binding of ^{125}I-rh·neuregulinβ1 to MDA-MB-453 cells, as did rh·neuregulinβ1 (Fig. 4c), indicating that rr·NTAKα2a binds to the same receptor as rh·neuregulinβ1 does.

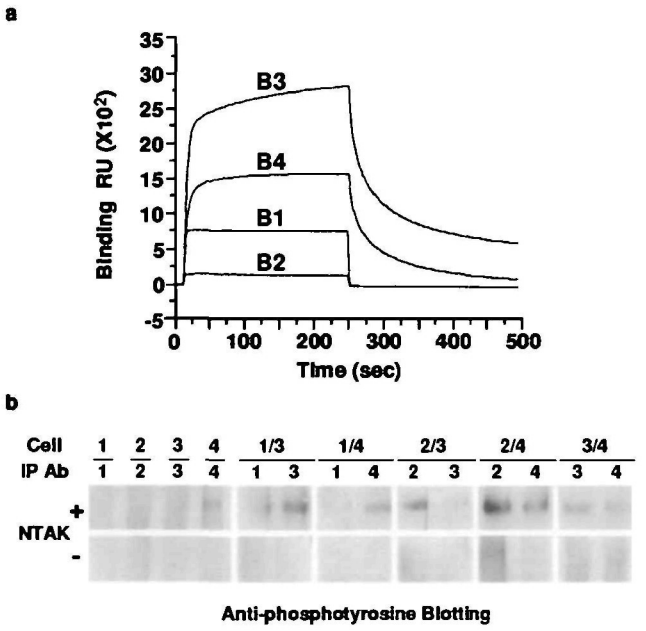

Fig. 3. **NTAK-ErbB interactions.** a: Sensorgrams of NTAK-ErbB interactions. The binding of purified rr·NTAKα2a to the purified ectodomain of ErbB was determined by surface plasmon resonance analysis with a BIAcore 2000 apparatus. b: Tyrosine phosphorylation of ErbBs by rr·NTAKα2a in 32D cells (1×10^6 cells) transiently expressing ErbB1-B4 (1, 2, 3, and 4 represent single expression, and 1/3, 1/4, 2/3, 2/4, and 3/4 pairwise expression). Untreated or rr·NTAKα2a-treated (100 ng/ml) transient transfectants were immunoprecipitated with ErbB antibodies (IP Ab 1-4) and then analyzed with anti-phosphotyrosine antibody PY-20 as described previously (*18*). One microgram of anti-ErbB1 (UBI), anti-ErbB2 (#Ab-1) (CALBIOCHEM), anti-ErbB3 (#841), or anti-ErbB4 (#C-18) (Santa Cruz Biotech.) was used.

Overall, NTAK is functionally similar to neuregulin in terms of directly activating ErbB4, transactivating ErbB1, B2, and B3, and stimulating the differentiation of MDA-MB-453 cells.

A nucleotide probe corresponding to the sequence of NTAK hybridized predominantly with 3.6, 5.0, and 7.2 kb transcripts in PC-12 cells (Fig. 5a). In adult rat tissues, these three NTAK transcripts were found in most parts of the brain, especially the olfactory bulb and cerebellum, and the 3.6 and 5.0 kb transcripts in the thymus. They were not detected in heart, adrenal gland, or testis. The tissue-specific expression pattern of NTAK mRNA differs from that of neuregulin mRNA, which is expressed in heart, liver, kidney, stomach, spinal cord, ovary, lung, spleen, and skin (*20, 27*).

In situ hybridization of rat E11.5 embryos with an NTAK-specific RNA probe demonstrated that NTAK mRNA is expressed in the telencephalon, but not in the hindbrain or heart expressing neuregulin mRNA (Fig. 5, b and c). The differences between the NTAK and neuregulin mRNA expression patterns in embryo and adult rats suggest different functional roles *in vivo*. It has been reported that ErbB3 is expressed in migrating neural crest

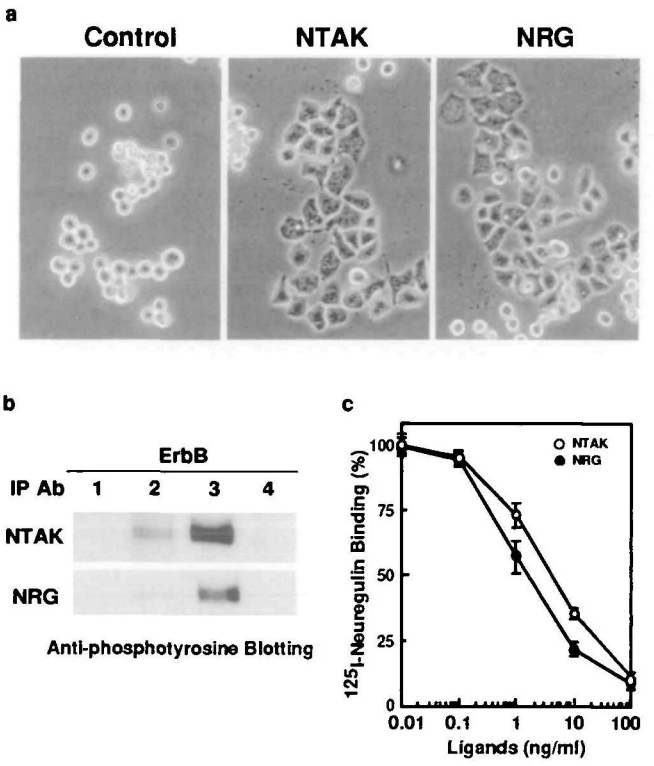

Fig. 4. **Biological effect of NTAK on MDA-MB-453 cells.** a: Induction of MDA-MB-453 cell differentiation. Cells were cultured for 24 h in DMEM supplemented with 2% FCS. rr·NTAKα2a (20 ng/ml) or rh·neuregulinβ1 (20 ng/ml) (gifts from Dr. Richard Vandlen, Genentech) was added to the medium and then the incubation was continued for an additional 3 days. b: Tyrosine phosphorylation of ErbB in MDA-MB-453 cells induced by rr·NTAKα2a (20 ng/ml) or rh·neuregulinβ1 (5 ng/ml). c: Competitive inhibition of the binding of ^{125}I-rh·neuregulinβ1 to MDA-MB-453 cells. Recombinant h·neuregulinβ1 was labeled with ^{125}I-Bolton-Hunter reagent (ICN) (*31*). Binding competition assays were carried out as described previously (*15*). Each point is the mean of quadruplicate measurements.

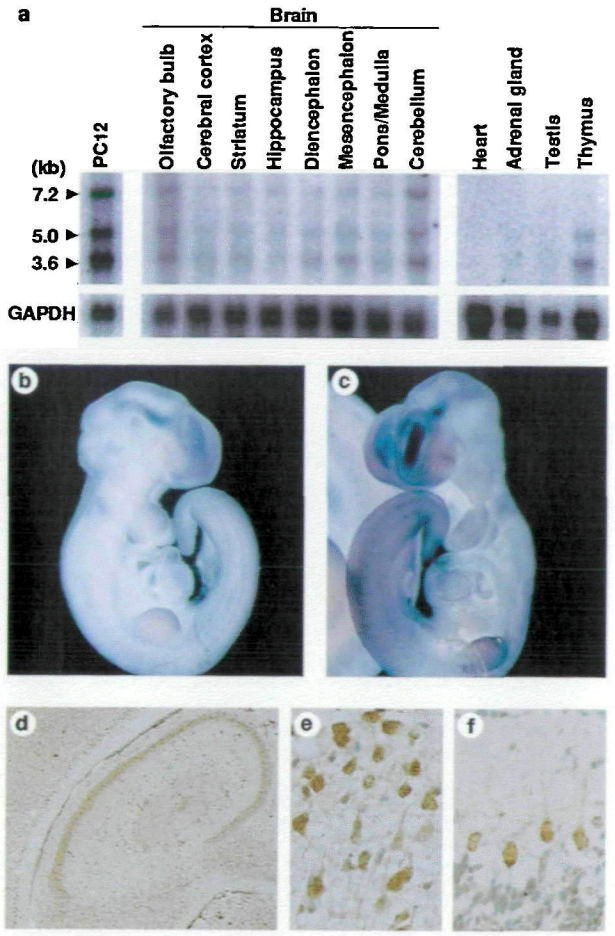

Fig. 5. **mRNA and protein detection of NTAK.** a: Northern blotting analyses. Poly(A)$^+$ RNA preparations of PC12 cells (2.5 μg) and 4-week-old rat tissues (brain, 5.0 μg; heart, 7.5 μg; adrenal gland, 4.0 μg; testis, 2.0 μg; and thymus, 6.0 μg) were immobilized on Hybond N$^+$ (Amersham). b-c: Whole mount *in situ* hybridization of a rat E11.5 embryo with an NTAK-specific probe (a *Sal*I/*Not*I 1,530 bp fragment) labeled with digoxygenin. b, lateral; c, ventral. d-f: Immunohistostaining of NTAK in a 4-week-old rat brain (horizontal section). NTAK protein was positively stained (brown color) in the pyramidal layer of the hippocampus (d, ×20), pyramidal cells in the occipital cortex of the cerebrum (e, ×100), and Purkinje cells in the cerebellum (f, ×100).

cells of the head and trunk, and that ErbB4 is expressed in the hindbrain (*8*). The distinct expression patterns of NTAK, ErbB3, and ErbB4 indicate paracrine signaling *in vivo*. However, the ligand-receptor relationship between NTAK and the ErbB family must be examined more precisely.

In a 4-week old rat, immunostaining of the NTAK protein was widely distributed in the neurons of the whole brain, consistent with the mRNA expression pattern. The most intense staining was found in the pyramidal layer of the hippocampus (Fig. 5d). Pyramidal cells of the cerebral cortex (Fig. 5e) and Purkinje cells of the cerebellum (Fig. 5f) were also stained positively. Most of the NTAK-positive cells were neurofilament-positive and GFAP-negative on double immunostaining (Higashiyama *et al.*, unpublished observation), suggesting NTAK was mainly produced by neuronal cells.

In summary, we have cloned a new member of the EGF family, NTAK, that shows limited structural homology to neuregulins and other EGF-like ligands, but which has the same biological properties as neuregulins. Thus, NTAK is neuregulin-like rather than EGF-like. However, the distributions of NTAK and neuregulins differ, suggesting that there may be multiple mechanisms for activation of the ErbB protein kinase family in the central nervous system.

We acknowledge Dr. M. Klagsbrun (Harvard Medical School) for reviewing of the manuscript, and Drs. S. Nomura and V.V. Shuvaev (Osaka University) for *in situ* hybridization and surface plasmon resonance analyses, respectively. S. Higashiyama is deeply grateful to Tasuku and Yumiko Higashiyama for their continuous encouragement.

REFERENCES

1. Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Libermann, T.A., Schlessinger, J., Downward, J., Mayes, E.L.V., Whittle, N., Waterfield, M.D., and Seeburg, P.H. (1984) Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* **309**, 418-425
2. Coussens, L., Yang-Feng, T.L., Liao, Y.-C., Chen, E., Gray, A., McGrath, J., Seeburg, P.H., Libermann, T.A., Schlessinger, J., Francke, U., Levinson, A., and Ullrich, A. (1985) Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. *Science* **230**, 1132-1139
3. Plowman, G.D., Whitney, G.S., Neubauer, M.G., Green, J.M., McDonald, V.L., Todaro, G.J., and Shoyab, M. (1990) Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. *Proc. Natl. Acad. Sci. USA* **87**, 4905-4909
4. Plowman, G.D., Culouscou, J.-M., Whitney, G.S., Green, J.M., Carlton, G.W., Foy, L., Neubauer, M.G., and Shoyab, M. (1993) Ligand specific activation of HER4/p180^{erbB4}, a fourth member of the epidermal growth factor receptor family. *Proc. Natl. Acad. Sci. USA* **90**, 1746-1750
5. Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R.C., Barnard, J.A., Yuspa, S.H., Coffey, R.J., and Magnuson, T. (1995) Targeted disruption of mouse EGF receptor: Effect of genetic background on mutant phenotype. *Science* **269**, 230-234
6. Sibilia, M. and Wagner, E.F. (1995) Strain-dependent epithelial defects in mice lacking the EGF receptor. *Science* **269**, 234-238
7. Miettinen, P.J., Berger, J.E., Meneses, J., Phung, Y., Perdersen, R.A., Werb, Z., and Derynck, R. (1995) Epithelial immaturity and multiorgan failure in mice lacking epidermal growth factor receptor. *Nature* **376**, 337-341
8. Meyer, D. and Birchmeier, C. (1995) Multiple essential functions of neuregulin in development. *Nature* **378**, 386-390
9. Gassmann, M., Casagranda, F., Orioli, D., Simon, H., Lai, C., Klein, R., and Lemke, G. (1995) Aberrant neural and cardiac development in mice lacking the ErbB4 neuregulin receptor. *Nature* **378**, 390-394
10. Lee, K.-F., Simon, H., Chen, H., Bates, B., Hung, M.-C., and Hauser, C. (1995) Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* **378**, 394-398
11. King, C.R., Kraus, M.H., and Aaronson, S.A. (1985) Amplification of a novel v-*erb*B-related gene in a human mammary carcinoma. *Science* **229**, 974-976
12. Cohen, S. (1964) Isolation and biological effects of an epidermal growth-stimulating protein. *Nat. Cancer Inst. Monograph* **13**, 13-27
13. Derynck, R., Roberts, A.B., Winkler, M.E., Chen, E.Y., and Goeddel, D.V. (1984) Human transforming growth factor-α: precursor structure and expression in *E. coli*. *Cell* **38**, 287-297
14. Shoyab, M., Plowman, G.D., McDonald, V.L., Bradley, J.G., and Todaro, G.J. (1989) Structure and function of human amphiregulin: a member of the epidermal growth factor family. *Science*

243, 1074-1076
15. Higashiyama, S., Abraham, J.A., Miller, J., Fiddes, J.C., and Klagsbrun, M. (1991) A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF. *Science* **251**, 936-939
16. Shing, Y., Christofori, G., Hanahan, D., Ono, Y., Sasada, R., Igarashi, K., and Folkman, J. (1993) Betacellulin: a mitogen from pancreatic β cell tumors. *Science* **259**, 1604-1614
17. Toyoda, H., Komurasaki, T., Uchida, D., Takayama, Y., Isobe, T., Okuyama, T., and Hanada, K. (1995) Epiregulin, a novel epidermal growth factor with mitogenic activity for rat primary hepatocytes. *J. Biol. Chem.* **270**, 7495-7500
18. Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B.J., Sela, M., and Yarden, Y. (1996) Diversification of Neu differentiation factor and epidermal growth factor signaling by combinatorial receptor interactions. *EMBO J.* **15**, 2452-2467
19. Riese II, D.J., Raaij, T.M., Plowman, G.D., Andrewa, G.C., and Stern, D.F. (1995) The cellular response to neuregulins is governed by complex interactions of the erbB receptor family. *Mol. Cell. Biol.* **15**, 5770-5776
20. Wen, D., Peles, E., Cupples, R., Suggs, S.V., Bacus, S.S., Luo, Y., Trail, G., Hu, S., Silbiger, S.M., Levy, R.B., Koski, R.A., Lu, H.S., and Yarden, Y. (1992) Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. *Cell* **69**, 559-572
21. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988) Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487-491
22. Higashiyama, S., Lau, K., Besner, G.E., Abraham, J.A., and Klagsbrun, M. (1992) Structure of heparin-binding EGF-like growth factor. *J. Biol. Chem.* **267**, 6205-6212
23. Malmqvist, M. (1993) Biospecific interaction analysis using biosensor technology. *Nature* **361**, 186-187
24. Simpson, R.J., Smith, J.A., Moritz, R.L., O'Hare, M.J., Rudland, P.S., Morrison, J.R., Lloyd, C.J., Grego, B., Burgess, A.W., and Nice, E.C. (1985) Rat epidermal growth factor: complete amino acid sequence. *Eur. J. Biochem.* **153**, 629-637
25. Chang, H., Riese, II, D.J., Gilbert, W., Stern, D.F., and McMahan, U.J. (1997) Ligands for ErbB-family receptors encoded by a neuregulin-like gene. *Nature* **387**, 509-512
26. Carraway III, K.L., Weber, J.L., Unger, M.J., Ledesma, J., Yu, N., Gassmann, M., and Lai, C. (1997) Neuregulin-2, a new ligand of ErbB3/ErbB4-receptor tyrosine kinases. *Nature* **387**, 512-516
27. Wen, D., Suggs, S.V., Karunagaran, D., Liu, N., Cupples, R.L., Luo, Y., Janssen, A.M., Ben-Baruch, N., Trollinger, D.B., Jacobsen, V.L., Meng, S.-Y., Lu, H.S., Hu, S., Chang, D., Yang, W., Yanigahara, D., Koski, R.A., and Yarden, Y. (1994) Structural and functional aspects of the multiplicity of Neu differentiation factors. *Mol. Cell. Biol.* **14**, 1909-1919
28. Marquardt, H., Hunkapiller, M.W., Hood, L.E., and Todaro, G.J. (1984) Rat transforming factor type I: structure and relation to epidermal growth factor. *Science* **223**, 1079-1082
29. Kimura, H., Fischer, W.H., and Schubert, D. (1990) Structure, expression and function of a schwannoma-derived growth factor. *Nature* **348**, 257-260
30. Abraham, J.A., Damm, D., Bajardi, A., Miller, J., Klagsbrun, M., and Ezekowitz, R.A.B. (1993) Heparin-binding EGF-like growth factor: characterization of rat and mouse cDNA clones, protein domain conservation across species, and transcript expression in tissues. *Biochem. Biophys. Res. Commun.* **190**, 125-133
31. Holmes, W.E., Sliwkowski, M.X., Akita, R.W., Henzel, W.J., Lee, J., Park, J.W., Yansura, D., Abadi, N., Raab, H., Lewis, G.D., Shepard, H.M., Kuang, W.-J., Wood, W.I., Goeddel, D.V., and Vandlen, R.L. (1992) Identification of heregulin, a specific activator of $p185^{erbB2}$. *Science* **256**, 1205-1210