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David W. Barnes

Serum-free mouse embryo (SFME) cells, which were derived from 16-day-old Balb/c mouse embryo brain, grow in absence of serum without losing genomic normality or proliferative potential, and require epidermal growth factor (EGF) for normal growth. EGF is a well studied mitogen that binds to a specific receptor on the cell surface membrane to activate the proliferative signal transduction pathways. The activated receptor is a tyrosine specific protein kinase, and tyrosine phosphorylation is one of the important mediators of EGF receptor (EGFR) signal transduction.

Using anti-phosphotyrosine Western immunoblotting, we detected a 100 kDa protein which is tyrosine-phosphorylated in response to EGF in SFME cells. This protein is constitutively phosphorylated in an SFME cell line which expresses the *neu* oncogene. The *neu* oncogene encodes an analog protein of EGFR which does not require a ligand for activation, and *neu*-transformed SFME cells are tumorgenic in mice.This protein, p100 was not a fragment of EGFR, and was not antigenically related to other signal transduction phosphoproteins of about 100 kDa. We attempted to purify p100 from *neu* SFME tumor cells for amino acid sequencing.

Purification and Identification of a 100 kDa Protein, Which is Tyrosine-Phosphorylated by EGF Stimulation in SFME Cells

by

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Major Professor, representing Biochemistry and Biophysics

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Chair of Department of Biochemistry and Biophysics

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Dean of Graduate School

I understand that my thesis will be become part of the permanent collection of Oregon State University libraries. My signature below authorized release of my thesis to any reader upon request.

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Kaoru Murayama, Author

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Chapter 1. Introduction

Serum-free mouse embryo (SFME) cells, which were derived from 16-day-old Balb/c mouse embryo brain, grow in the absence of serum without losing genomic normality or proliferative potential, unlike conventional mouse embryo cells in serum-supplemented media. SFME cells are cultured in a rich basal medium supplemented with insulin, transferrin, epidermal growth factor (EGF), high density lipoprotein (HDL), and fibronectin (Loo et al., 1987). SFME cells *in vitro* express the intermediate filament protein nestin (Loo et al., 1994), a marker for neuroepithelial stem cells *in vivo* (Lendahl et al., 1990), and express astrocytic markers, including glial fibrillary acidic protein and glutamine synthetase, when exposed to transforming growth factor beta or cytokines acting through gp130 (Loo et al., 1995; Nishiyama et al., 1993; Sakai et al., 1990; Solem et., 1990; Weisz et al., 1993). Cells with properties similar in some respects to SFME can be isolated from human embryonic brain (Loo et al., 1991).

SFME cells require EGF for normal growth, and die within 48 hours without EGF (Loo et al., 1987; Rawson et al., 1990;

Abbreviations: SFME, Serum-free mouse embryo; EGF, epidermal growth factor; EGFR, EGF receptor; TGF, transforming growth factor; FGF, fibroblast growth factor; bFGF, basic FGF; SH2, Src homology 2; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-triphosphate; PKC, protein kinase C; PY, phosphotyrosine.

Rawson et al., 1991). Transforming growth factor alpha (TGF-alpha), which also binds to EGF receptor, can substitute EGF although it is not as effective as EGF, and fibroblast growth factor (FGF) can partially replace EGF (Loo et al., 1989). On the other hand, SFME cell proliferation is reversibly inhibited by serum or platelet-free plasma (Loo et al., 1987). SFME cells transformed with the human *Ha-ras* or rat *neu* oncogene or expressing the protooncogene *bcl-2* no longer require EGF for survival (Shirahata, et al., 1990; Loo, et al., 1997). The acute dependence of SFME cells on EGF and the ability to grow the cells in the absence of growth factors present in serum provides an uncomplicated way to examine EGF signaling and the relationship of growth factor signaling to apotosis *in vitro*.

EGF is a well-studied mitogen of 53 amino acids with molecular mass of 6 kDa. Although the early studies revealed that EGF stimulates the proliferation of epithelial cells *in vivo* (Carpenter and Cohen, 1990), subsequently numerous biological activities on various tissues have been reported, some of them apparently unrelated to mitogenesis (see the list by Carpenter an Wahl, 1990; also refer to Fisher and Lakshmanan, 1990). EGF also shows mitogenic activity for various types of cultured cells *in vitro*.

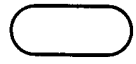
Is has been suggested that EGF plays an important role in brain development. Neurogenesis in the mammalian central nervous system (CNS) mostly occurs during embryonic development, and ends shortly after birth (Reynolds and Weiss, 1992). EGF receptor (EGFR) is known to be expressed in mouse embryo brain, and its expression increases dramatically during day 15 to 19. (Adamson and Meek, 1984). Many *in vitro* studies indicate that EGF has various effects on CNS nerve cells, such as proliferation of embryonic and neonatal

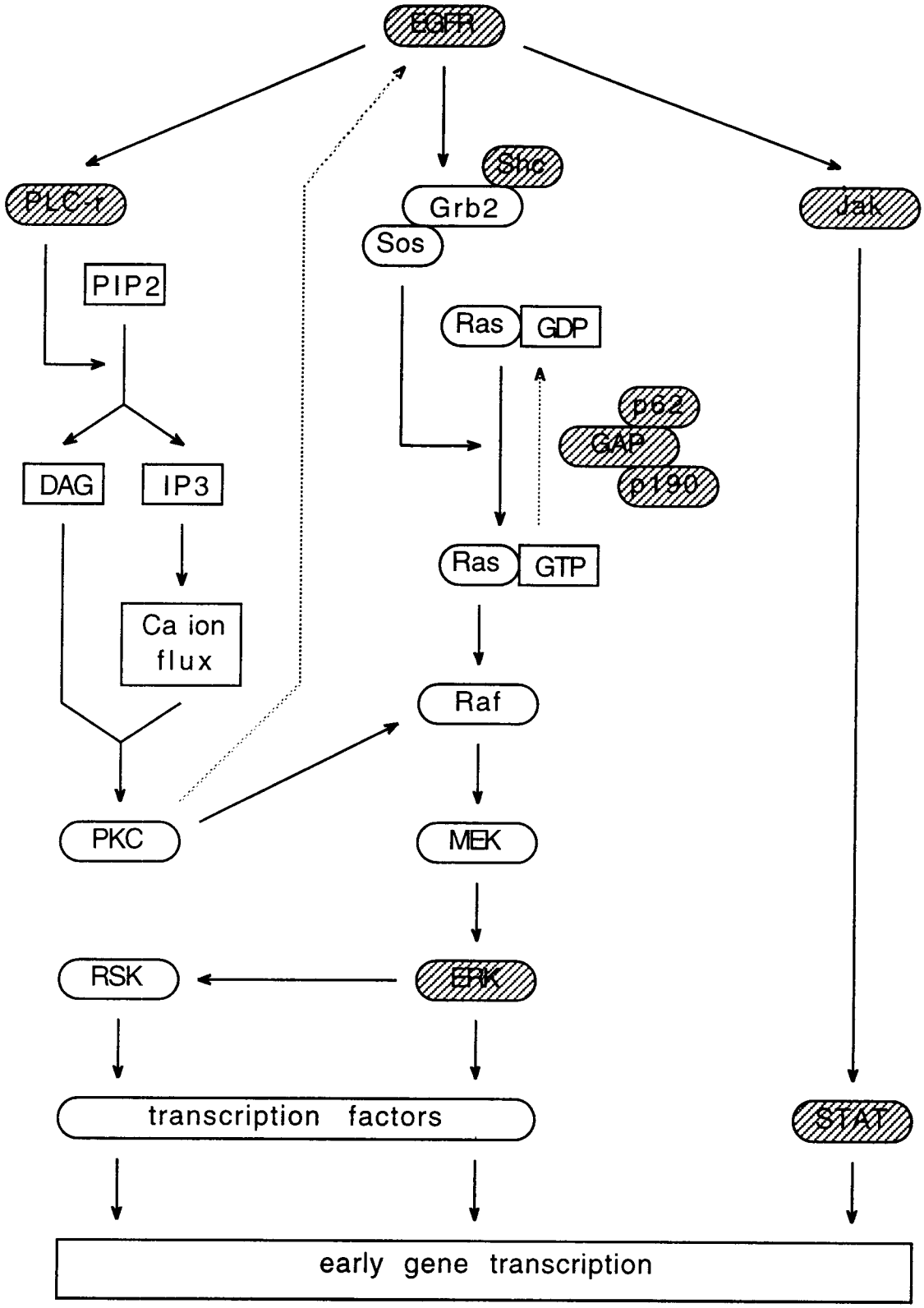
retinal cells of rat (Anchan et al., 1991), generation of neurons and astrocytes from mouse embryonic striatal cells (Reynolds et al., 1992) and adult striatal cells (Reynolds and Weiss, 1992), and mitogenic activity in the olfactory epithelium of neonatal rat (Mahanthappa and Schwarting, 1993). However, mechanisms by which growth factors, including EGF, promote brain development *in vivo* are still to be studied.

EGF binds tightly to a specific transmembrane receptor on the cell surface membrane (Carpenter and Cohen, 1990; Fisher and Lakshmanan, 1990). The EGFR, or c-*erbB-1* gene product, is a 170 kDa glycoprotein, divided into three domains: a cysteine-rich extracellular domain to which the ligand binds, a transmembrane domain which consists of hydrophobic amino acids, and a cytoplasmic domain which has tyrosine kinase activity. The cytoplasmic domain is also divided into two parts; a tyrosine kinase domain and a carboxyl-terminal tail with tyrosine residues which are autophosphorylated subsequent to the ligand binding. Phosphorylation of tyrosine residues is one of the most important mediators in signal transduction, not only of EGFR, but also of many pathways of other growth factors (Glenney, 1992).

Generally, the EGF monomer is considered to bind to EGFR monomer in stoichiometry of 1 : 1, and this binding causes a conformational change of the receptor resulting in dimerization and kinase activation, although there is a report that one EGF molecule binds to two receptor molecules (Lemmon and Schlessinger, 1994). The kinase of each monomer in the receptor dimer phosphorylates the other on tyrosine residues at the carboxyl termini, and also phosphorylates several other cytoplasmic proteins. The phosphotyrosine

Figure 1. Signal transduction pathways of EGFR

See the text for abbreviations.  : non-protein molecules;  : non-phosphotyrosine proteins;  : phosphotyrosine proteins; ..... : negative regulations.


EGFR
PLC-γ
PIP2
DAG
IP3
Ca ion flux
PKC
Shc
Grb2
Sos
Ras GDP
p62
GAP
p190
Ras GTP
Raf
MEK
ERK
RSK
Jak
STAT
transcription factors
early gene transcription

residues are recognized by proteins which have Src homology 2 (SH2) domains to activate proliferative pathways (Figure 1. Also see the reviews by Ullrich and Schlessinger, 1990; and Schlessinger and Ullrich, 1992).

The most well-studied pathway subsequent to receptor tyrosine kinase activation by ligand binding is the Ras/Raf/MEK/ERK pathway (See the reviews by Blenis, 1993; Marshall, 1995). One of the phosphotyrosine residues in the cytoplasmic domain of EGFR is recognized by the SH2 domain of the Grb2 adaptor protein, which constitutively binds to Sos, a Ras guanine nucleotide exchange factor (Egan et al., 1993; Ruff-Jamison et al., 1993a; Ravichandran et al., 1995). Ras is inactive when binding to GDP, and active when binding to GTP, and Sos activates Ras by exchanging GDP to GTP. Another adaptor protein Shc is known to bind to EGFR, and both EGFR-Shc-Grb2-Sos and EGFR-Grb2-Sos complexes have been reported (Buday and Downward, 1993; Sasaoka et al, 1994). On the other hand, GAP (GTPase activating protein) works as negative regulator, enhancing the GTPase activity of Ras. GAP and its two associated proteins, p62 and p190 are known to be tyrosine-phosphorylated through EGF-dependent mechanism (Ellis et al., 1990; Cantley et al., 1991). Active Ras-GTP recruits cytoplasmic protein Raf to the plasma membrane, and Raf is activated by a still unknown factor. Although the mechanism of Raf activation is unclear, phosphorylation primarily of serine residues is thought to be involved (Daum et al., 1994). Raf itself is also a serine kinase and activates MEK (MAP kinase/ERK-activating kinase), which is a "dual-specificity protein kinase", activating another protein, ERK (extracellular signal-regulated kinases) by phosphorylating threonine and tyrosine residues. ERK, which is a serine/threonine kinase, enters the nucleus, and phosphorylates transcription factors for early gene

expression. In addition, cytoplasmic protein RSK (ribosomal protein S6 kinase) is also activated by ERK through serine and/or threonine phosphorylation, and enters the nucleus to stimulate early gene transcription.

A second pathway involves phospholipase C-gamma (PLC-gamma). The PLC-gamma molecule has two src homology 2 (SH2) domains, and binds to the cytoplasmic domain of EGFR. PLC-gamma, activated by EGFR through tyrosine-phosphorylation, cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) in the cell membrane to generate diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG is an activator of protein kinase C (PKC), and IP_3 induces calcium ion flux from endoplasmic reticulum (ER) to cytoplasm (Ullrich and Schlessinger, 1990; Schlessinger and Ullrich, 1992).

PKC is a serine/threonine kinase, and plays complex roles in signal transduction. It is known that PKC negatively regulates EGFR through threonine phosphorylation (Morrison et al., 1996). However, at present, at least 11 members of PKC have been reported. These can be divided in to three classes: classical PKC (cPKC) including PKC-alpha, beta I, beta II and gamma; novel PKC (nPKC) including PKC-delta, epsilon, eta and theta; and atypical PKC (aPKC) including PKC-zeta and iota/lambda. Each member shows different characteristics. For example, cPKCs are Ca^{2+} dependent, but nPKCs are not, and aPKCs are not activated by Ca^{2+} or DAG (Stabel and Parker, 1991; Akimoto et al., 1996). There have been reports that cPKCs activate Raf as positive regulators of the EGFR signal transduction (Sozeri et al., 1992; Kolch et al., 1993). Therefore, more detailed studies in each PKC member seem to be necessary.

Recently, a distinctively different Jak-STAT pathway was proposed. This pathway was originally discovered in studies of interferon (IFN)-alpha and gamma signal transduction (See the reviews by Darnell et al., 1994; Ihle et al., 1994). Jak (Janus kinase) proteins have tyrosine kinase function as indicated by their name, and also must be phosphorylated on their own tyrosine residues for enzyme activation. STAT (signal transducers and activators of transduction) proteins have SH2 domains to recognize phosphotyrosine, and are tyrosine-phosphorylated by active Jaks. In the example of IFN-alpha, when the ligand binds to its receptor, two types of Jak proteins, Jak1 and Tyk2 bind to the cytoplasmic domain of the receptor. Tyrosine-phosphorylated active Jak1 and Tyk2 phosphorylate tyrosine residues of STAT1 and STAT2 respectively. STAT1 and STAT2 are translocated into the nucleus where they form a heterodimer through each other's SH2 domains and phosphotyrosine residues. This heterodimer binds to a specific ISRE (interferon-stimulated response element) sequence in early genes, involving another DNA binding protein p48. In the case of IFN-gamma, Jak1 and Jak2 bind to the receptor, then STAT1 homodimer binds to GAS (IFN-gamma activation site) without p48. At present, at least 3 Jaks (Jak1, Jak2 and Tyk2) and 4 STATs (STAT1, STAT2, STAT3 and STAT4) have been reported and Jak1, STAT1 and STAT3 are known to be involved in EGFR signal transduction. However, most of the details of this pathway are still to be studied, including whether some other accessary proteins are involved.

Other possible pathways exist for EGF signal transduction. Phosphatidylinositol 3-kinase (PI3K) has been reported to bind to EGFR, and be activated through tyrosine-phosphorylation. However, this is still controversial because EGFR lacks the specific sequence in its carboxyl tail for PI3K binding (Cantley et al., 1991; Soltoff et al, 1994). In

addition, two other EGFR substrates, Eps8 and Eps15 have been reported, the involvement of these proteins in EGF-related pathways remain to be elucidated (Fazioli et al., 1993a and b; Alvarez et al., 1995).

Using Western blotting with anti-phosphotyrosine monoclonal antibody, we detected a protein with molecular mass approximately 100 kDa which is tyrosine-phosphorylated in SFME cells in response to EGF. This protein is constitutively phosphorylated, with or without EGF, in a transgenic SFME cell line which expresses the *neu* oncogene (Shirahata, et al., 1990) and is tumorigenic in mice.

The *neu* oncogene produces an analog protein of the EGFR gene although they are distinct genes (See the review by Dougall et al., 1994). The *neu* oncogene codes for a 185 kDa protein and is oncogenically activated from the protooncogene (cellular *neu* or c-*neu*, also called c-*erbB*-2) by a point mutation. It is known that amplification and overexpression of c-*neu* (c-*erbB*-2) as well as mutation to its oncoform can lead to malignant transformation (Guy et al., 1992 and 1996), and similar results are obtained with *EGFR* (c-*erbB*-1) (Mendelsohn and Gabriolove, 1995; Stumm et al., 1996). The physiological function of Neu in normal cell growth and tissue development is still unclear. There have been reports of Neu expression in fetal and adult tissues, including fetal brain.

Currently, at least 4 members of the EGFR family have been identified: EGFR(ErbB-1), neu(ErbB-2), ErbB-3, and ErbB-4. Like the EGFR, the Neu onco and protooncoproteins have a extracellular domain, a transmembrane domain (the site of the point mutation), and a cytoplasmic domain which has tyrosine kinase activity (Dougall, et al., 1993). The point mutation

in the transmembrane domain allows dimerization and tyrosine kinase activation of the oncoprotein without ligand. It has been discovered that EGFR family members can form heterodimers, and properties of these receptors such as ligand binding, signal transduction, and physiological function, now seem to be more complex than previously thought.

Several potential ligands for the Neu protooncoprotein have been reported, but the relationships among these ligands and members of the receptor family are not yet clear. Neither of the EGFR ligands, EGF or TGF-alpha bind to Neu. Heregulin, originally thought to be a Neu ligand is more likely a ligand of Erb-3 and -4. Results are complicated because Neu can be activated through heterodimerization with ErbB-3 or -4 (Lemmon and Schlessinger, 1994).

Difference in signal transduction between EGFR and Neu has been suggested because of low homology of amino acid sequence between the carboxyl-terminal tails for the two proteins. These tails are important for substrate binding. However, no specific substrate for either has been reported. There is a possibility that EGFR/Neu heterodimer activates a pathway distinct from that of either the EGFR or Neu homodimers.

In this project, we characterized the 100 kDa protein, which is tyrosine-phosphorylated specifically by EGF stimulation, and attempted to purify and identify by amino acid sequencing. This is a first step toward precisely defining the role of this protein in EGF signaling pathways and the mechanisms of EGF action in brain development and oncogenesis.

Chapter 2. Materials and Methods

1. Materials

1.1 Cell culture and cell lysis

Dulbecco's modified Eagle medium (D-MEM), F-12 nutrient mixture (Ham), and HEPES were obtained from Gibco BRL/Life Technologies (Gaithersbug, MD). Sodium bicarbonate and sodium selenite were obtained from Fisher Scientific (Fair Lawn, NJ). Penicillin, streptomycin, ampicillin, bovine insulin, human transferrin, heparin, trypsin, soybean trypsin inhibitor, and other chemicals were obtained from Sigma Chemical (St. Louis, MO). High-density lipoprotein (HDL) was prepared as described by Gospodarowicz (1984) and Loo et al. (1989). Mouse epidermal growth factor (EGF) and human basic fibroblast growth factors (bFGF) were obtained from Upstate Biotechnologies (Lake Placid, NY). Fetal bovine serum (FBS) was obtained from HyClone Laboratories (Logan, UT).

Nonident P-40, sodium ortho-vanadate, all protease inhibitors including aprotinin, pepstatin A, phenylmethylsulfonylfluoride (PMSF), leupeptins and antipain, and other chemicals were obtained from Sigma Chemicals.

1.2 SDS-PAGE, Western blotting and silver staining

The apparatus for electrophoresis and Western blotting for 8 x 10 cm (H x W) gels were obtained from Bio-Rad Laboratories (Hercules, CA).

Acrylamide (acrylamide : N-N'-methylenebisacrylamide = 29:1), sodium dodecyl sulfate (SDS), ammonium persulfate,

N,N,N',N'-tetramethylenediamine (TEMED), protein molecular weight standards, and Silver Stain Kit for gel silver staining were obtained from Bio-Rad Laboratories. Bromophenol blue (BPB) was obtained from Sigma Chemical. Polyvinylidene difluoride (PVDF) membrane for Western immunoblotting was obtained from Millipore (Bedford, MA).

Anti-phosphotyrosine monoclonal antibody, sheep anti-human EGF receptor (EGFR) polyclonal antibody, rabbit anti-human STAT1 polyclonal antibody, and rabbit anti-human B-Raf polyclonal antibody were obtained from Upstate Biotechnologies. Goat anti-mouse IgG horseradish peroxidase conjugate and Goat anti-rabbit IgG horseradish peroxidase conjugate were obtained from Bio-Rad Laboratories. Rabbit anti-sheep IgG horseradish peroxidase conjugate was obtained from Cappel (West Chester, PA). ECL kit and Hyperfilm-MP were obtained from Amersham Life Science (Cleveland, OH).

1.3 Protein purification

Anti-phosphotyrosine Immunoaffinity Purification Kit was obtained from Upstate Biotechnologies. Centriflo CF50A for protein concentration was obtained from Amicon (Beverly, MA). Thioglycolic acid was obtained from Sigma. SYPRO Orange protein dye used to estimate the proteins' positions in polyacrylamide gels was obtained from Bio-Rad. For protein elecctroelution form polyacrylamide gels, I used the Elutrap kit by Schleicher and Schuell (Keene, NH).

1.4 Preparation for amino acid sequencing

3-[cyclohexalamino]-1-propanesulfonic acid (CAPS) was obtained from Sigma Chemical. Coomassie Brilliant Blue (CBB) G-250 was obtained from Bio-Rad.

2. Methods

2.1 Cell culture

SFME cells were derived from the neural stem cells of 16-day-old Balb/c embryo and grown in the serum-free culture medium as described by Loo *et al.* (1987, 1989). The serum-free culture medium was prepared as below: a one-to-one mixture of D-MEM and Ham's f-12 was supplemented with 1.5 mM HEPES, pH 7.4, 1.2 g/L sodium bicarbonate, 200 U/ml penicillin, 200 µg/ml streptomycin and 25 µg/ml ampicillin (F12:DME). Immediately before the cells were plated, F12:DME was supplemented with 10 µg/ml bovine insulin, 40 µg/ml human transferrin, 10 µg/ml human HDL, 10 nM sodium selenite and 50 ng/ml mouse EGF (supplemented serum-free medium).

For Western immunoblotting, the cells were replated on 30 mm dishes in the density of 1 x 10^6 cells in 2 ml of supplemented serum-free medium per dish (with or without EGF). The cells were grown under the following conditions before cell lysis: for 5 hours without EGF or bFGF (-EGF,-bFGF), for 5 hours with EGF (+EGF, -bFGF), for 5 hours without EGF or bFGF followed by 10 minutes with EGF (-/+EGF, -bFGF), for 5 hours with FGF (-EGF, +bFGF), or for 5 hours without EGF or bFGF followed by 10 minutes with FGF(-EGF,-/+bFGF). Heparin was also added to the medium when bFGF was added in order to enhance the effect of bFGF (Loo et al.,

1989); the concentrations of bFGF and heparin were 10 ng/ml and 1 µg/ml respectively.

NIH 3T3 cells were grown in 10 % FBS in F12:DME. For Western blotting, the cells were replated in 30 mm dishes containing F12:DME with 10 % FBS (1 x 10^6 cells /2 ml), and were harvested 5 hours later.

A431 human carcinoma cell lysate was supplied by Upstate Biotechnology as a positive control of human STAT1 and human B-Raf.

2.2 Transgenic SFME cells

SFME cells were transfected with human *Ha-ras* or rat *neu* oncogene as described by Shirahata et al. (1990).

2.3 Cell lysis

The lysis buffer for extraction of cytoplasmic proteins was 20 mM Tris-HCl, 100 mM NaCl, 20 mM sodium ortho-vanadate, 1.0 % Nonident P-40, pH 7.4. Protease inhibitors (5 µg/ml aprotinin, 1 µg/ml pepstatin A, 1 µg/ml PMSF, 1 µg/ml leupeptins, and 1 µg/ml antipain) were added to the lysis buffer immediately before cell lysis (Sambrook, et al. 1989).

The lysis buffer was spread on the dishes immediately after the medium was removed (200 µl buffer/1 x 10^6 cells/30 mm dish). Lysate from each dish was collected in an Eppendolf tube after lysis was confirmed microscopically. Optimal lysis occurred after 1-2 minutes. The tubes were centrifuged at 12,000 x g for 10 minutes to remove cellular debris, and the supernatants were transferred to fresh tubes.

The lysates were stored at -80 degree C. Samples were thawed and again centrifuged at 12,000 x g for 10 minutes before fractionation by SDS-PAGE.

2.4 SDS-PAGE, Western blotting and silver staining

SDS-PAGE gels were generated using 375 mM Tris-HCl, 6.0 % acrylamide (acrylamide : N-N'-methylenebisacrylamide = 29:1), 0.1 % SDS, 0.05 % ammonium persulfate, 0.5 % TEMED, pH 8.8. The stacking gel was 125 mM Tris-HCl, 4.0 % acrylamide, 0.1 % SDS, 0.05 % ammonium persulfate, 1.0% TEMED, pH 6.8. Samples were prepared in 50 mM Tris-HCl, 1.0 % SDS, 0.1 % BPB, 10 % glycerol, 100 mM dithiothritol (DTT), pH 6.8, and boiled for 5 minutes. Electrophoresis was performed in running buffer containing 25 mM Tris-HCl, 192 mM glycine, 0.1 % SDS, pH 8.3, at 30 mA/gel. For separation of large proteins, the samples were run until prestained marker proteins of approximate 50 kDa migrated to the bottom of the gels. Buffer for Western blotting was 25 mM Tris-HCl, 192 mM glycine, 20 % methanol, pH 8.3. Proteins were blotted onto PVDF membrane at 100 V for 1 hour or at 30 V overnight. The blotted membrane was washed with phosphate buffered saline (PBS) containing 0.05 % Tween-20 (Washing Buffer), and blocked with 1.0 % BSA in Washing Buffer for anti-phosphotyrosine immunostaining, or with 3.0 % dried skim milk in Washing Buffer for anti-EGFR, anti-Stat1, or anti-B-Raf immunostaining. The membrane was incubated with the primary antibody at the vendor's recommended dilution, and with the secondary antibody at 1:6000 dilution in 1.0 % BSA in Washing Buffer. Excess antibodies were washed out after each incubation. Western blot signals were detected with an ECL kit according to the manufacturer's directions.

Silver staining was also performed according to the manufacturer's directions.

2.5 Protein electroelution from acrylamide gels

Samples were concentrated with Centriflo CF50A to approximately 200 µl. SDS-PAGE was performed as described for Western blotting except that the samples were prepared in a sucrose sample buffer (62.5 mM Tris-HCl, 2.0 mM EDTA, 3.0 % SDS, 100 mM sucrose, 100 mM DTT, pH 6.9), and heated at 60 degree C for 15 minutes, instead of the glycerol buffer and boiled for 5 minutes. In addition 0.1 mM thioglycolic acid was added on the top of the running buffer before the samples were applied to scavenge reactive compounds that would interfere with amino acid sequencing.

Position of proteins in the polyacrylamide gels were estimated by molecular size markers stained for 20 minutes with SYPRO Orange in the SDS-PAGE running buffer, and area of the gel containing each protein were cut out and placed in an Elutrap chamber. The proteins were eluted in buffer containing 15 mM NH_4CO_3, 0.1 % SDS, pH 8.2, for 4 hours, following directions supplied by Schleicher and Schuell.

2.6 Preparation for Amino acid sequencing

Samples were concentrated by lyophilizing. SDS-PAGE was performed as described for protein purification. Electroblotting was performed as described for Western blotting except a CAPS buffer (10 mM CAPS, 10 % methanol, pH 11.0) was used instead of the Tris-glycine buff
blotted PVDF membranes were stained in 0.25 % CBB

methanol, and partially destained in 50 % methanol to visualize the proteins.

Chapter 3. Results

1. Detection and characterization of p100

SFME cells were grown in 5 different conditions: without EGF or bFGF for 5 hours (-EGF, -bFGF); with EGF for 5 hours (+EGF, -bFGF); without EGF or bFGF for 5 hours followed by 10 minutes with EGF (-/+EGF, -bFGF); with bFGF (-EGF, +FGF); and without EGF or bFGF for 5 hours followed by 10 minutes with bFGF (-EGF, -/+bFGF). Cytoplasmic proteins were extracted as described in Materials and Methods. Each sample was boiled for 10 minutes immediately after cell lysis to inactivate residual proteases and analyzed by anti-phosphotyrosine(PY) Western immunoblotting. The result is shown in Figure 2. No difference was observed among the three conditions: -EGF,-bFGF (lane 1); -EGF, +FGF (lane 4); and -EGF, -/+bFGF (lane 5). In contrast, +EGF, -bFGF (lane 2) and -/+EGF, -bFGF (lane 3) showed two growth factor-dependent bands: one at approximately 170 kDa, which is the size of EGFR, and the other at 100 kDa.

A similar experiment was performed with human *Ha-ras* and rat *neu* oncogene transformed SFME cells. The result of *ras* SFME cells is shown in Figure 3; *ras* SFME cells showed additional bands not seen in untransformed SFME cells, but significant differences were not observed among the 5 different conditions used to identify growth factor-dependent phosphorylation. Although some response to EGF could be seen in lane 3 (-/+EGF, -bFGF), it was much weaker than the response of untransformed SFME cells (Figure 2, lane 3). Phosphorylation of p100 was difficult to evaluate in *ras* SFME cells because many fine bands appeared between 80 and 120 kDa.

Figure 2. Anti-phosphotyrosine Western immunoblot of untransformed SFME cells. Samples were boiled for 10 minutes to inactivate proteases immediately after cell lysis. Samples were adjusted to represent equal cell numbers in all lanes. Numbers indicate molecular mass references. EGFR and p100 are indicated with arrows.

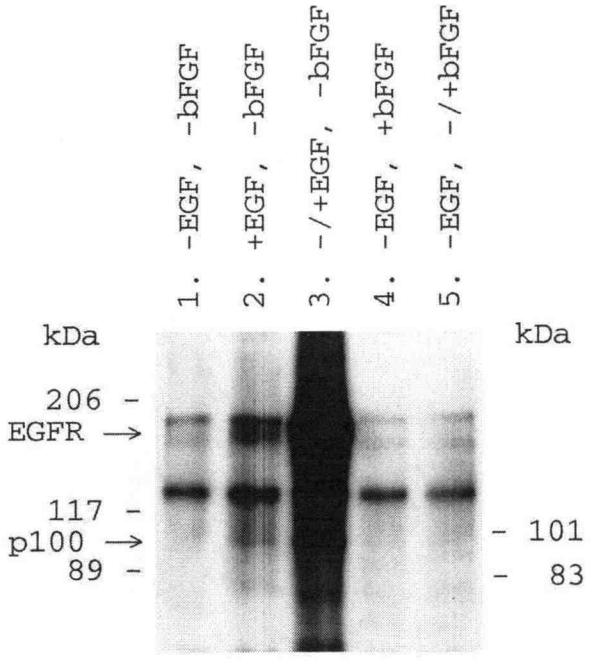
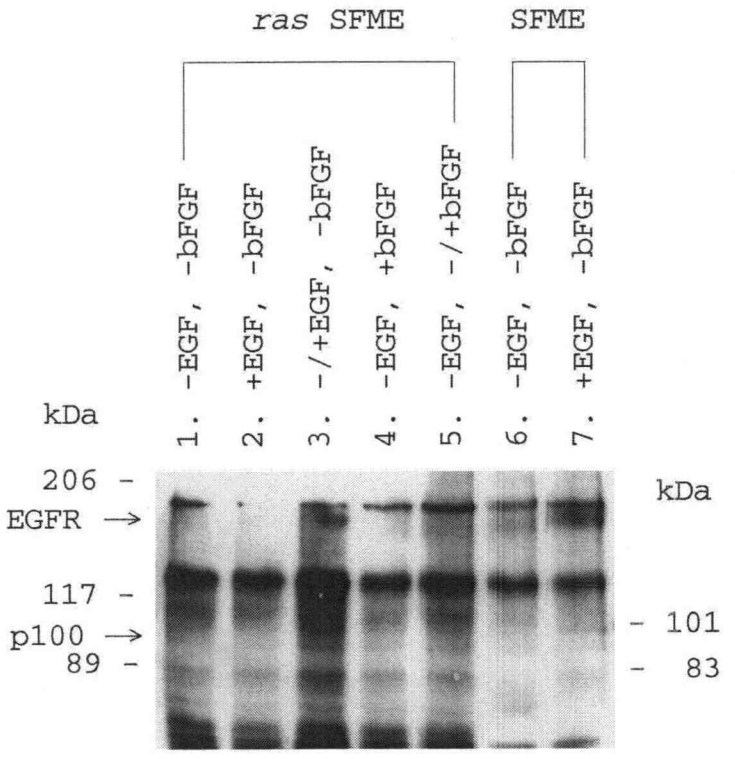


Figure 3. Anti-phosphotyrosine Western immunoblot of *ras* oncogene transformed SFME cells. Samples were boiled for 10 minutes to inactivate proteases immediately after cell lysis. Samples were adjusted to represent equal cell numbers in all lanes. Numbers indicate molecular mass references. EGFR and p100 are indicated with arrows.



Results with *neu* SFME cells are shown in Figure 4. *neu* SFME cells in all 5 conditions showed an SFME-like pattern when cultured -/+EGF, -FGF (lane 3, Figure 2). Additionally, a strong band was detected above the EGFR band. We assumed that this band is the Neu oncoprotein, p185. The EGFR and p100 were detected in all conditions with or without EGF. The EGFR appeared a little stronger in lane 2 (+EGF, -bFGF), and more so in lane 3 (-/+EGF, -bFGF). The p100 band also appeared a little stronger in lane 3, indicating a response to EGF.

After ligand binding, the EGFR is internalized and transported to lysosomes for degradation (Carpenter and Wahl, 1990) and proteolytic degradation products of the EGFR have been observed in other laboratories in experiments similar to those described above. Therefore it was necessary to examine the possibility that p100 was a fragment of EGFR. We performed Western immunoblotting of the samples with an anti-human EGFR polyclonal antibody that recognizes the cytoplasmic domain of EGFR and has cross reactivity with mouse EGFR. The anti-EGFR antibody detected only a band at 170 kDa, consistent with our identification of the band as the EGFR and suggesting that proteolytic degradation was successfully avoided in our protocol (Figure 5) and that p100 is not a fragment of EGFR.

The time course of tyrosine phosphorylation in p100 was examined. SFME cell were lysed after 5, 10, and 15 minutes after EGF administration, and compared on a Western blot. Figure 6 shows that tyrosine phosphorylation reaches a maximum at about 5 minutes, then declines.

Our results indicated that p100 might be a protein which is tyrosine-phosphorylated through activation of the EGFR

Figure 4. Anti-phosphotyrosine Western immunoblot of *neu* oncogene transformed SFME cells. Samples were boiled for 10 minutes to inactivate proteases immediately after cell lysis. Samples were adjusted to represent equal cell numbers in all lanes. Numbers indicate molecular mass references. EGFR, Neu and p100 are indicated with arrows.

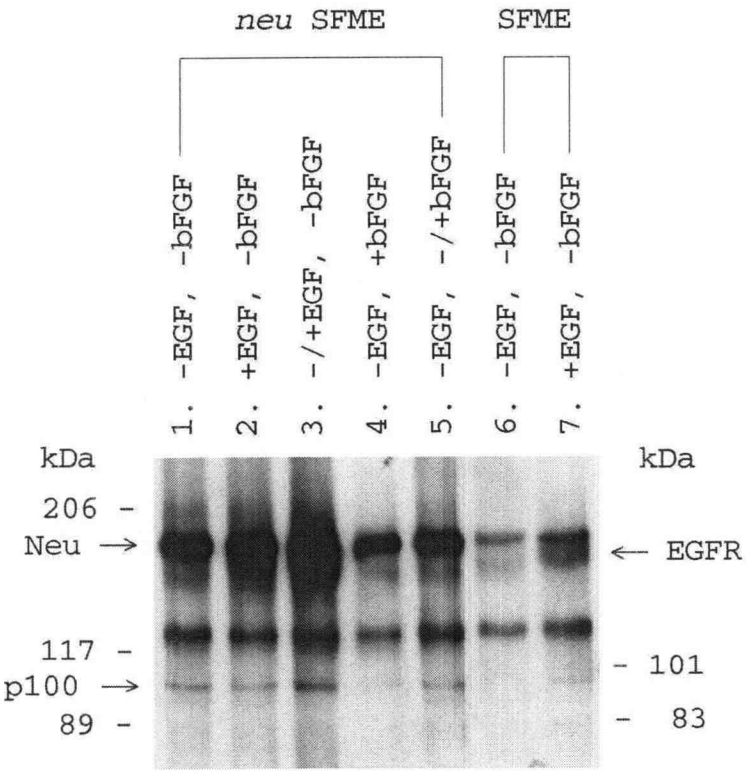


Figure 5. Anti-EGF receptor Western immunoblot of untransformed and *neu* oncogene transformed SFME cells. Samples were boiled for 10 minutes to inactivate proteases immediately after cell lysis. Samples were adjusted to represent equal cell numbers in all lanes. Numbers indicate molecular mass references. EGFR is indicated with an arrow.

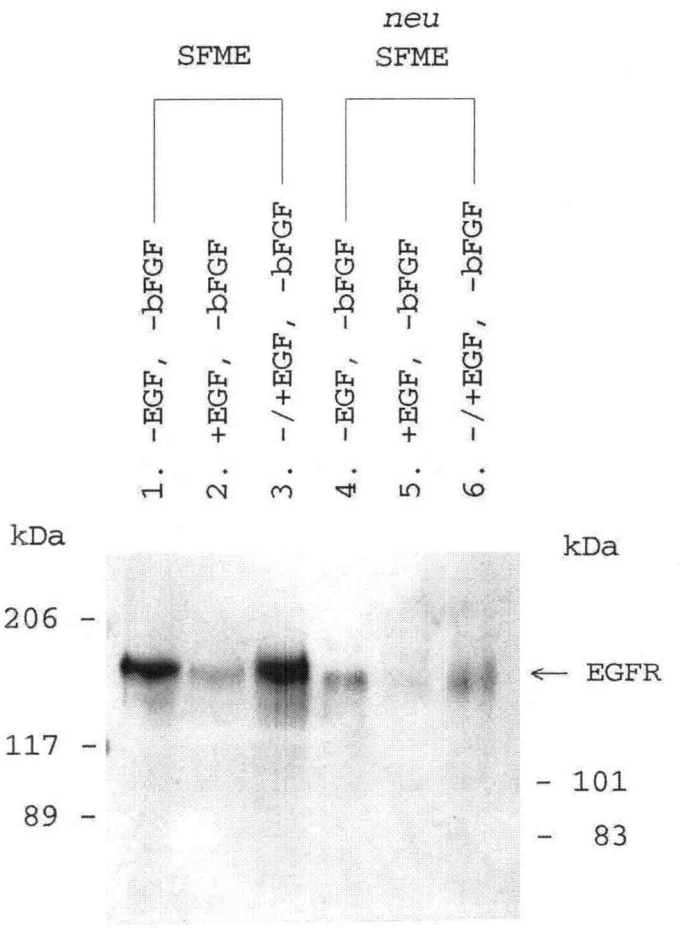
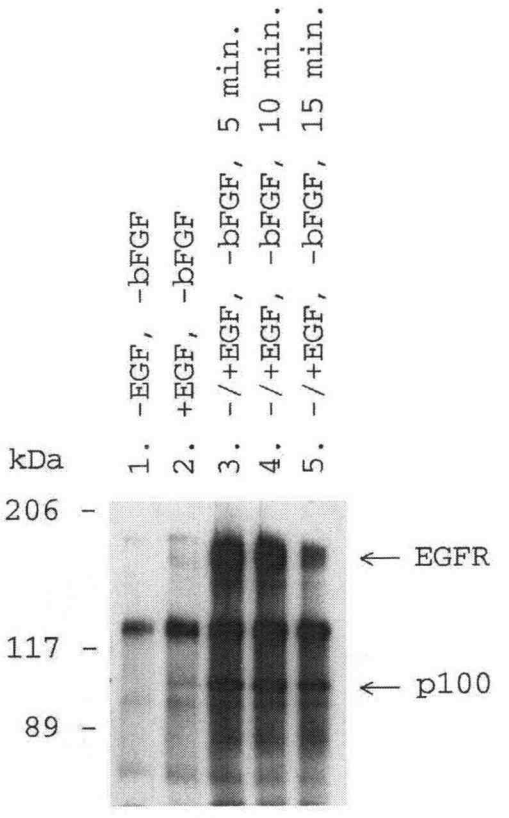


Figure 6. Time course of tyrosine phosphorylation by EGF stimulation in untransformed SFME cells. Samples were not boiled after cell lysis. Samples were adjusted to represent equal cell numbers in all lanes. Numbers indicate molecular mass references. EGFR and p100 are indicated with arrows.



(and possibly also Neu), and we postulated that the protein may be involved in EGF signal transduction within the cell. A search of databases and the literature for EGF-dependent tyrosine-phosphorylated proteins did not identify a previously studied candidate for p100 observed in other laboratories. Thus, we decided to purify and identify this protein by amino acid sequencing.

2. Tumor production to harvest p100

A major problem was encountered in our attempts to purify the protein. The p100, like many other candidate signal proteins, exists at low concentration in cells, and it became evident that isolation of the protein from cultured SFME cells in amounts sufficient for primary structure analysis was not practical. To provide sufficient material for the protein isolation, we used the procedure described below.

The approximately $1x10^7$ *neu* SFME cells suspended in F12:DME medium (total volume about 200 µl) were injected under the epidermis of the left shoulder of a Balb/c moused. The mouse was killed 2-3 weeks later when the *neu* SFME cells formed a visible tumor, and the tumor was excised for protein extraction as described below.

After fatty tissue and blood were washed out with F12:DME medium, the *neu* tumor was minced and incubated in F12:DME medium containing 50 ng/ml EGF for 5 minutes. The tumor cells were centrifuged and medium removed. The cell pellet was then incubated in the lysis buffer described above. The tumor was extracted at 4 degree C overnight, then the buffer was removed the next day. This procedure was repeated five times (five days) to complete protein extraction. For these preparations, we did not boil the

extracts, because a major portion of the proteins in the extracts, including phosphotyrosine proteins, were precipitated by the boiling.

We compared the *neu* tumor extract and untransformed SFME and *neu* SFME cell extracts by anti-phosphotyrosine Western immunoblot (Figure 7). The tumor extract (lane 5) showed a very similar pattern to *neu* SFME cells in -/+EGF, -bFGF condition (lane 4), except the two bands at 130-140 kDa and 90 kDa appeared stronger in the tumor extract. Because no additional bands were detected near p100, we concluded that this *neu* tumor extract could be used for p100 purification. We used three Balb/c mice for tumor production.

3. p100 purification

Our purification procedure involved three steps: Step 1, the lysis buffer adjustment; Step 2, anti-PY immunoaffinity chromatography; and Step 3, separation of the protein by preparative SDS-PAGE and electroelution.

In Step 1 we varied the concentration of salt, chelator, detergent and tyrosine phosphatase inhibitors, starting with the formulation suggested by the vendor for the Anti-phosphotyrosine Immunoaffinity Purification Kit. This buffer contains 20 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 1 % Nonident P-40, 0.2 mM sodium ortho-vanadate (Na_3VO_4), pH 7.4. Ortho-vanadate is a inhibitor of phosphatases, and we raised its concentration to 20 mM for additional protection of phosphotyrosine residues without adverse effect on the extraction. Our goal was maximal extraction of the EGF-responsive p100 with minimal extraction of constitutively phosphorylated phosphotyrosine proteins.

Figure 7. Anti-phosphotyrosine Western immunoblot of untransformed and *neu* oncogene transformed SFME cells, and *neu*-SFME tumor cell extract. Samples were <u>not</u> boiled after cell lysis. Samples of the untransformed and *neu* SFME cells were adjusted to represent equal cell numbers in all lanes. Numbers indicate molecular mass references. EGFR, Neu and p100 are indicated with arrows.

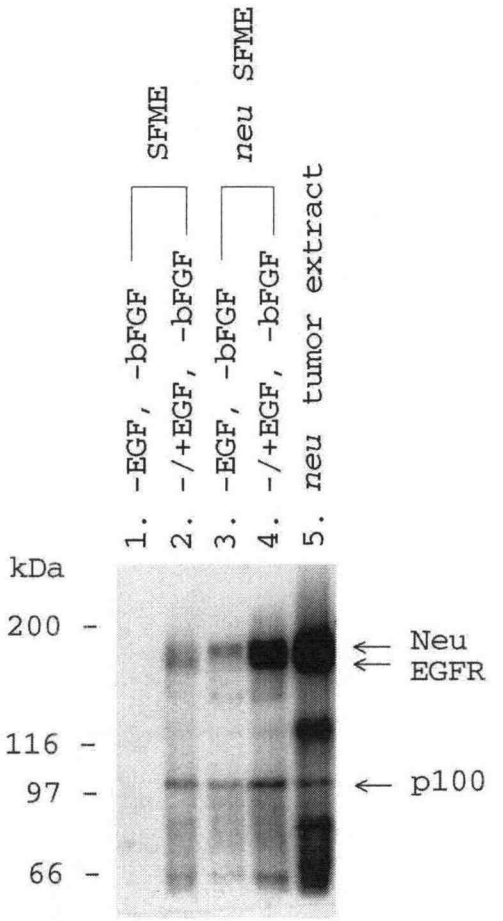
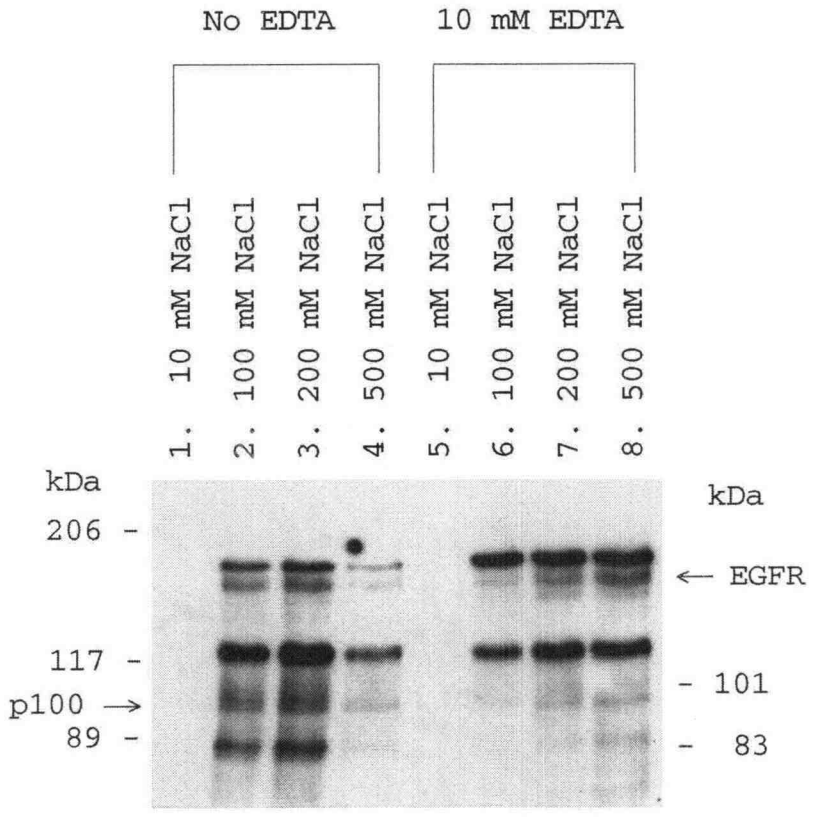


Figure 8. Anti-phosphotyrosine Western immunoblot of untransformed SFME cells (+EGF, -bFGF) in different NaCl concentration and with or without 10 mM EDTA. Samples were boiled for 10 minutes to inactivate proteases immediately after cell lysis. Samples were adjusted to represent equal cell numbers in all lanes. Numbers indicate molecular mass references. EGFR and p100 are indicated with arrows.

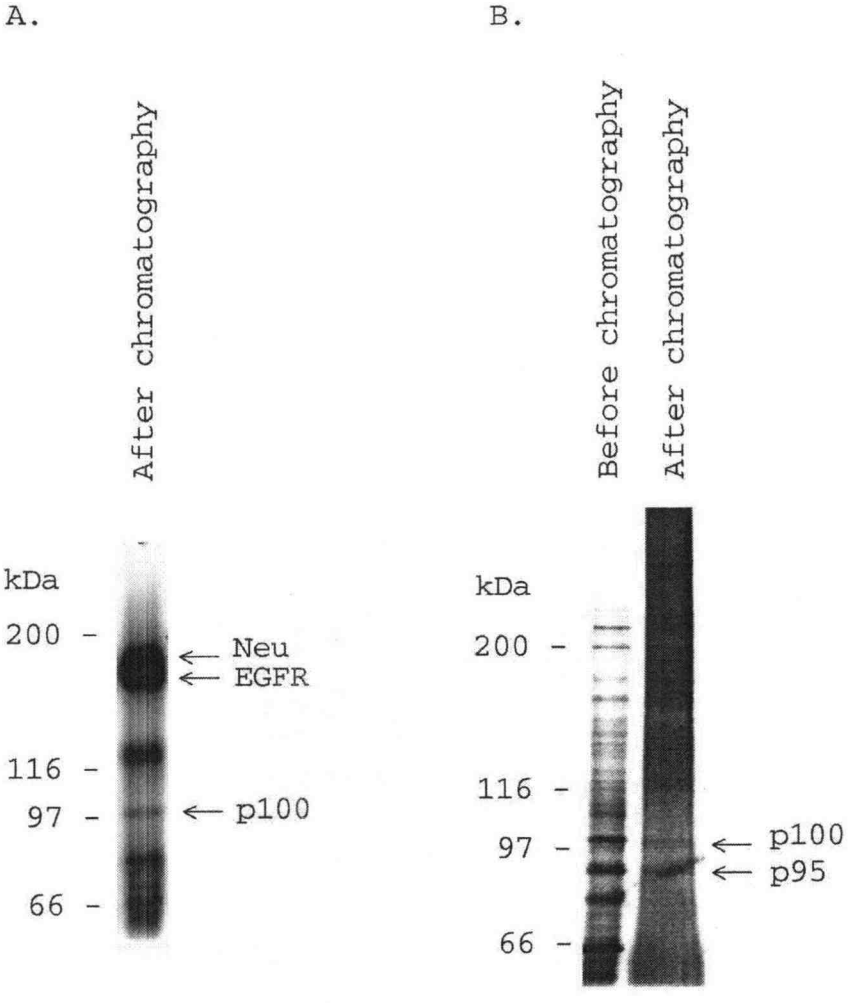


Final results of experiments examining effects of changes in concentration of NaCl and EDTA on phosphoprotein extraction are shown in Figure 8. Here we examined extraction of untransformed SFME (+EGF, -bFGF) using anti-PY Western blotting. The samples were boiled for 10 minutes immediately after the cell lysis. We examined samples extracted with or without 10 mM EDTA, and with 10, 100, 200 or 500 mM NaCl. 100 or 200 mM NaCl without EDTA (lanes 2 and 3, respectively) showed the strongest p100 signal. We chose 100 mM NaCl because we were concerned that further raising the NaCl concentration might affect protein binding in the immunoaffinity chromatography. Removing EDTA had no effect on the chromatography. The final formula of the lysis buffer was 20 mM Tris-HCl, 100 mM NaCl, 1 % Nonidet P-40, 20 mM Na_3VO_4, pH 7.4.

Anti-PY immunoaffinity chromatography was performed as suggested by the vendor of the column material. The lysate was loaded on the column followed by washing with the lysis buffer, and phosphotyrosine proteins were eluted with 100 mM phenylphosphate in the lysis buffer. Binding and elution were confirmed with anti-PY Western blot.

The eluate fractions were pooled, and the phosphoprotein band pattern compared by anti-PY Western immunoblotting and in silver staining. As seen in Figure 9, protein stain and phosphoprotein signal showed similar patterns, indicating a major purification of phosphotyrosine proteins had occurred, and p100 appeared as a discrete band in silver staining. We also noticed another discrete band at approximately 95 kDa, which showed almost the same intensity as p100 in silver staining, although it was barely detected in the phosphotyrosine immunoblot. This protein is likely minimally phosphorylated, and may be a dephosphorylation product of p100. It is well established that proteins change mobility in

Figure 9. *neu* tumor extract before and after anti-phosphotyrosine affinity chromatography. A: anti-phosphotyrosine Western immunoblot of eluate after chromatography. B: silver staining of samples before and after chromatography. EGFR, Neu, p100 and p95 are indicated with arrows.



gel electrophoresis with the degree of tyrosine phosphorylation (Huang et al., 1995; Yan and Templeton, 1994). For these reasons, we decided to isolate p95 as well as p100.

The next step was designed to separate p100 and p95 from the other phosphotyrosine proteins in the eluate. The eluate was concentrated with Centriflo CF50A, then run on SDS-PAGE. The gels were stained with SYPRO Orange protein dye, which does not interfere with subsequent electroblotting or electroelution, and has relatively high sensitivity. Even after concentration, the amounts of p100 and p95 were not high enough to be visualized with the dye. Therefore we estimated the positions of these proteins in the gel using stained molecular mass references. Areas containing both proteins in the gels was cut out, and electroeluted as described in Materials and Methods. Purification was monitored with Western blotting and silver staining (Figure 10), and indicated that both proteins had been successfully isolated with minimal contamination.

4. Two candidates of p100: STAT1 and B-Raf

While carrying out this project, we identified two candidates for p95 or p100 from the literature. One is STAT1 (p91), which is known to be tyrosine-phosphorylated through EGF stimulation (Fu and Zhang, 1993; Ruff-Jamison et al., 1993; Silvennoien et al., 1993). We performed Western blotting using anti-human STAT1 polyclonal antibody which recognizes the carboxyl terminal of STAT1 and has cross-reactivity with mouse. Figure 11 shows the result of analyzing the anti-PY immunoaffinity chromatography eluate by anti-PY and anti-STAT1 Western blot. All samples were loaded

Figure 10. p100 and p95 after separation in SDS-PAGE and electroelution. A: anti-phosphotyrosine Western immunoblot. B: silver staining.

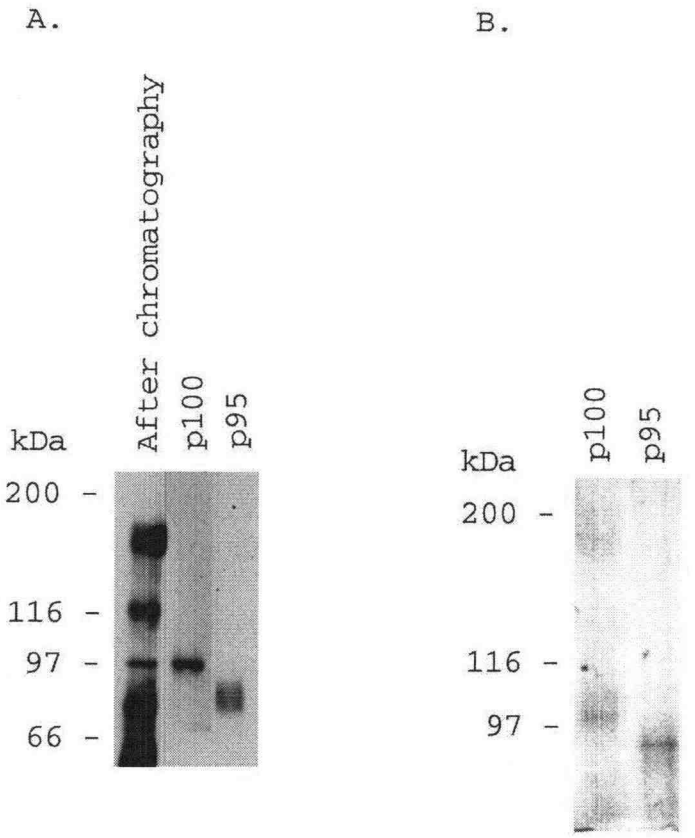
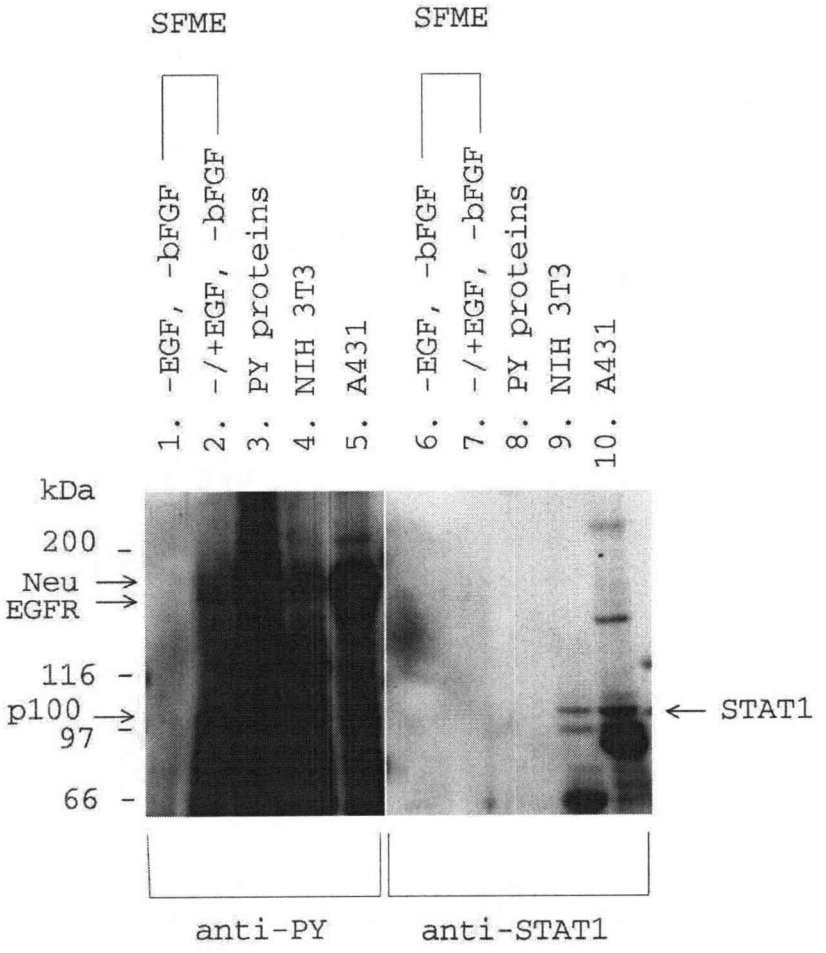


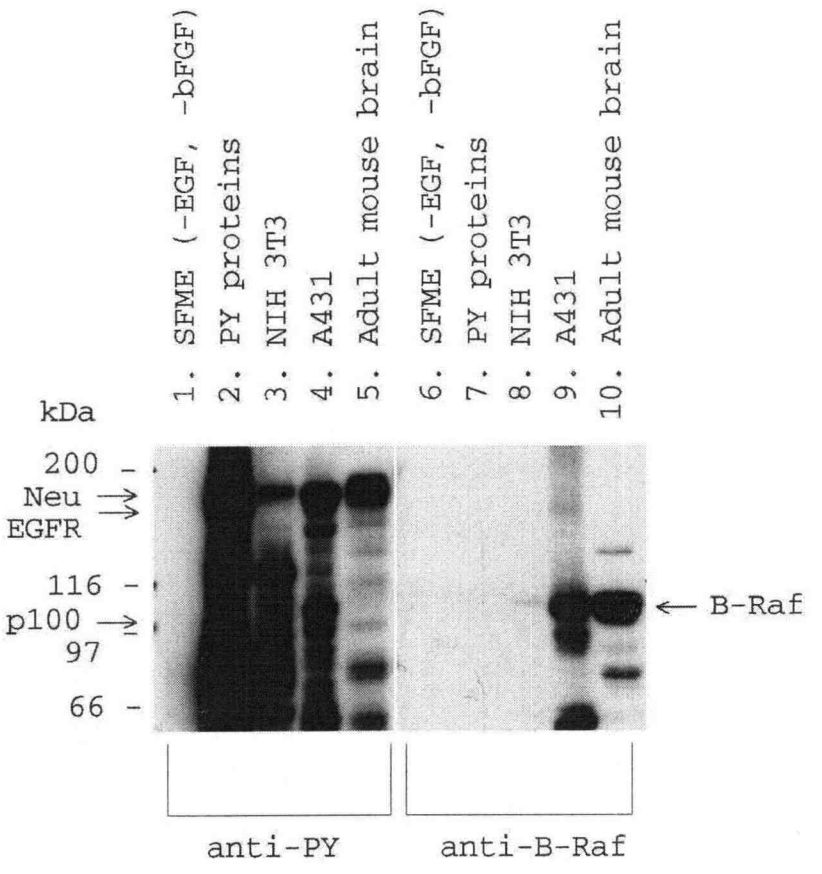
Figure 11. Anti-phosphotyrosine(PY) and anti-STAT1 Western immunoblots of identical samples. Identical amounts of each sample were loaded for both blots. Samples were <u>not</u> boiled after cell lysis. Samples were adjusted to represent equal cell numbers of SFME and NIH 3T3 cells. All samples were loaded on one gel. After SDS-PAGE and electroblotting, the PVDF membrane was cut in halves. The left half was immunostained for anti-PY, and the right immunostained for anti-STAT1. Numbers indicate molecular mass references. EGFR, Neu, p100 and STAT1 are indicated with arrows.



on one gel, and the same amount of each sample was used for both blotting. After SDS-PAGE and electroblotting, the PVDF membrane was cut in half, and one set of samples was stained with anti-PY, while the other was stained with anti-STAT1 antibody. A431 and NIH 3T3 are positive controls of human and mouse STAT1 respectively, and both showed a STAT1 signal. Other bands (possibly due to degradation or nonspecific binding) were seen, depending on tissue and cell line. Positive controls were as expected, but the PY protein eluate or crude extract of SFME cells with or without EGF showed no signal. We concluded that neither p100 nor p95 is STAT1, and that STAT1 is not tyrosine-phosphorylated through an EGF signal in SFME cells.

The other candidate is B-Raf. At present, at least 3 isoforms of Raf have been identified in vertebrates: c-Raf (p73), A-Raf (p68) and B-Raf (p94-97) (Daum et al., 1994). Storm et al. (1990) reported that B-*raf* is expressed in high levels especially in fetal brain, and adult cerebrum and testes while c-*raf* and A-*raf* are expressed ubiquitously in both fetal and adult tissues. Wixler et al. (1996) reported that B-Raf is activated in response to EGF. Although the main phosphorylation sites on Raf proteins are thought be serine residues, reports of tyrosine or threonine phosphorylation exist (Heidecker et al., 1992; Daum et al., 1994). We carried out Western blotting using anti-human B-Raf polyclonal antibody which recognizes the carboxyl terminal of B-Raf and has cross-reactivity with mouse. The result is shown in Figure 12. Anti-PY and anti-B-Raf Western blottings were performed as described for anti-STAT1 Western blotting. A431 and adult mouse brain extract are used as human and mouse B-Raf positive controls. Mouse brain was extracted with the lysis buffer used for SFME cells and *neu* tumors. Both A431 and mouse brain showed a strong signal at approximately 105

Figure 12. Anti-phosphotyrosine(PY) and anti-B-Raf Western immunoblots of identical samples. Identical amounts of each sample were loaded for both blots. Samples were not boiled after cell lysis. Samples were adjusted to represent equal cell numbers of SFME and NIH 3T3 cells. All samples were loaded on one gel. After SDS-PAGE and electroblotting, the PVDF membrane was cut in halves. The left half was immunostained for anti-PY, and the right immunostained for anti-B-Raf. Numbers indicate molecular mass references. EGFR, Neu, p100 and B-Raf are indicated with arrows.

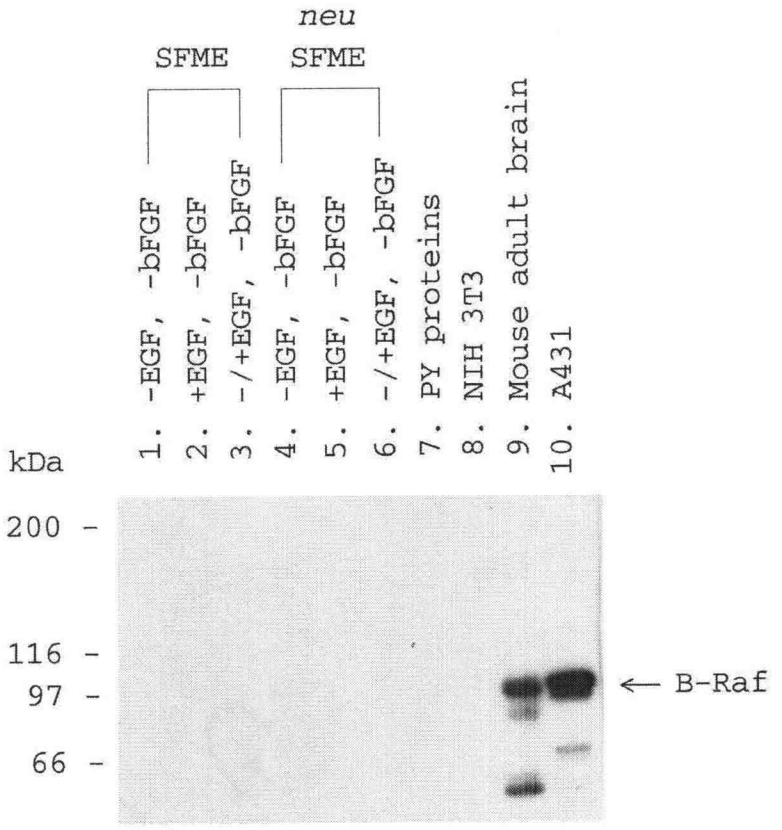


kDa, and we assumed this band to be B-Raf, because Moodie et al. (1994) reported that they detected B-Raf at 95-105 kDa. NIH 3T3 showed a fine signal at 105 kDa, but the PY protein eluate did not show this signal. We concluded that neither p100 nor p95 is B-Raf, and that B-Raf is not tyrosine-phosphorylated in respond to EGF in SFME cells. We also did not detect B-Raf in crude extracts of the untransformed and *neu* SFME cells, with or without EGF, despite our expectation that it might be present in these fetal brain derived cells (Figure 13). These results indicated that p95 or p100 might be novel EGF-responsive proteins, possibly involved in signal transduction, and protein sequencing should proceed.

5. Amino acid sequencing of p95 and p100

We were unable to sequence the samples through the facility of Oregon State University because of the small amount of material obtained (about 10 pmol). One sample was sent to a sequencing facility of Purdue University. Because we were uncertain if these proteins could be sequenced by methods and equipment available in most academic institutions, we first sent the less critical p95 electroeluate. However, p95 could not be sequenced because it could not be directly blotted on a PVDF membrane because of SDS in the electroelution buffer. The sample was returned to our laboratory, and electroblotted and visualized by CBB on a membrane as described in Materials and Methods. We sent the membrane to Purdue again, and it still could not be sequenced because of its amount, either because the amino acid terminal is blocked or because of the small amount of protein remaining after these procedures. However, because p95 could be visualized by CBB on the membrane after electroblotting, there is still a possibility that it can be sequenced. We are

Figure 13. Anti-B-Raf Western immunoblots. Samples were <u>not</u> boiled after cell lysis. Samples were adjusted to represent equal cell numbers of untransformed SFME, *neu* SFME and NIH 3T3 cells. Numbers indicate molecular mass references. B-Raf is indicated with an arrow.



searching for another institute which has more sensitive equipment.

Chapter 4. Discussion

1. Detection and characterization of p100

Using anti-phosphotyrosine (PY) Western immunoblotting we detected a protein with molecular size of approximately 100 kDa which is tyrosine-phosphorylated by EGF stimulation of SFME cells. The tyrosine phosphorylation of this protein, which we call p100, was EGF specific (not observed upon bFGF stimulation), occurred with or without EGF in *neu* oncogene transformed SFME cells. The *neu* oncogene produces an EGFR-analog protein which is constitutively active without ligand binding. Thus, we speculated that p100 could be a signal protein involved with either or both EGFR and Neu.

We examined also *ras* oncogene transformed SFME cells. However, because *ras* SFME cells showed many additional bands in Western blots than untransformed or *neu* SFME cells did, we couldn't determine if p100 is tyrosine-phosphorylated by EGF in these cells. Since Ras is in the middle of the signal transduction pathways, overexpression of active Ras may induce massive activation of downstream signal proteins. Ras activation may also lead to negative feedback regulation, such as threonine phosphorylation of EGFR and phosphatase activation by MAPK (Griswold-Prenner et al., 1993; Morrison et al., 1993) so that the whole picture is difficult to dissect.

We did not detect any bFGF specific signal in untransformed SFME cells by anti-PY Western blotting. It is established that bFGF is one of the FGFs which show angiogenic activity *in vivo*, as well as mitogenic activity in various types of mesoderm and neuroectoderm derived cells *in vitro*. Like EGFR, FGF receptor (FGFR) is a tyrosine kinase

receptor, and the ligand binding induces dimerization, tyrosine kinase activation and tyrosine phosphorylation at the carboxyl tail. Regarding signal transduction, it is known that FGFR activates PLC-gamma. At present, at least five FGFR genes have been identified, and each of them expresses proteins with various size, affinity to FGFR and tyrosine kinase activity. The receptors with high affinity and tyrosine kinase activity are in the range of 110-150 kDa (Burgess and Maciag, 1989; Jaye et al., 1992).

Although bFGF has shown mitogenic activity in SFME cells, it is not as effective as EGF (Loo et a., 1989). Probably FGFR is expressed and activated in SFME cells, but not sufficiently so to be detected in anti-PY Western blotting.

We suspected that p100 might be a degraded fragment of the EGFR because it was always strongly tyrosine-phosphorylated when the EGFR was phosphorylated and because after EGFR binding, the EGFR is transported to lysosome for degradation (Carpenter and Wahl, 1990). However, anti-EGFR polyclonal antibody did not recognize p100 or any other EGFR derived proteolytic products in our samples, suggesting that p100 is not the result of EGFR degradation. The EGFR signal was weaker with EGF in the untransformed and *neu* SFME cells because EGF accelerates the receptor's internalization and transportation to lysosome (Carpenter and Wahl, 1990). The EGFR seems to be expressed much less in *neu* SFME cells than the untransformed SFME cells. The active Neu oncoprotein and/or EGFR activated by heterodimerization with Neu may induce the feedback negative regulation.

We also found two candidates for p100 based on molecular size and EGF-responsive tyrosine phosphorylation. One of these, STAT1 (p91), is known to be tyrosine-phosphorylated

through EGF stimulation (Fu and Zhang, 1993; Ruff-Jamison et al., 1993b; Silvennoien et al., 1993). However, we did not detect STAT1 in the phosphotyrosine protein eluate of the anti-phosphotyrosine affinity chromatography, which contained only PY proteins Therefore, we concluded that p100 is not STAT1, and that STAT1 is not activated by EGF in SFME cells. Although we did not detect STAT1 in the crude extract of the untransformed SFME cells, with or without EGF, these experiments do not allow us to conclude if the SFME cells are expressing STAT1. Usually signal proteins exist in very small amount in cells, so sometimes they are difficult to detect with direct Western blotting using antibody to the protein, as we did in this project. This can be true even when using the very sensitive ECL detection method. Overloading samples may exceed the PVDF membrane's capacity, so, to determine if SFME cells are expressing STAT1, we would have to perform STAT1 immunoprecipitation before Western blotting.

The other candidate was B-Raf (p94-97). B-*raf* is reported to be expressed specifically in fetal brain as well as adult cerebrum and testes of normal mice, while c-*raf* and A-*raf*, which produce 73 kDa and 68 kDa proteins respectively, are expressed ubiquitously (Storm et al., 1990). This was especially of interest because SFME cells were derived from mouse embryonic brain. Although two serine residues are considered to be the major phosphorylation sites of B-Raf, there have been some reports of threonine or tyrosine phosphorylation (Heidecker et al., 1992; Daum et al., 1994). B-Raf kinase activity can be stimulated by EGF, although it is not clear if tyrosine phosphorylation is required in this activation (Wixler, et al. 1996). The phosphotyrosine protein eluate did not show a B-Raf signal, suggesting that p100 is not B-Raf, and that B-Raf is not tyrosine-phosphorylated by EGF in the SFME cells. We also did not detect B-Raf in crude extracts of the untransformed and *neu* SFME cells, with or

without EGF, despite our expectation that it might be present in these fetal brain derived cells. However, we can not be certain that SFME cells are expressing B-Raf for the same reason we described above for STAT1.

2. Tumor production to harvest p100

The p100 protein, like many other signal proteins, exists in very small amount in each cell so that harvesting enough for amino acid sequencing from SFME cells is not practical. Instead we turned to isolation from tumors derived from *neu* SFME cells injected under the epidermis of a Balb/c mouse. Tumor became visible 2-3 weeks after injection. Cytoplasmic proteins from the tumor cells were extracted with the same lysis buffer used for cultured SFME cells, and the anti-PY Western blot showed a very similar pattern to the neu SFME cells.

3. p100 purification

The lysis buffer was adjusted in the beginning of this project for maximal harvest of p100 while minimizing contamination with other phosphoproteins. In the beginning, we boiled the samples to ensure the protease and phosphatase inactivation; later we ceased boiling because it precipitated a significant amount of proteins including p100, and we also found that the enzyme inhibitors added in the lysis buffer seemed to be working sufficiently. We attempted to minimize NaCl concentration because this might affect p100 protein binding in the affinity chromatography, and also, because 500 mM NaCl can dissociate the nuclear membrane, releasing additional phosphoproteins as well as DNA and RNA, may

interfere with the chromatography. Because removing EDTA did not affect on the chromatography results, our final formula contained 100 mM NaCl without EDTA.

Once the lysis buffer formulation was determined, phosphotyrosine proteins were isolated from the *neu* tumor cell lysate using the anti-PY affinity chromatography. Purification was monitored by Western blotting and silver staining. In addition to p100, we detected a strong and discrete band at approximately 95 kDa, which is barely detected in anti-PY Western blot. We speculated that p95 has less phosphotyrosine residues than p100, and results as described above indicated that it is unlikely to be STAT1 or B-Raf. We do not know at this time if p95 tyrosine phosphorylation is EGF-specific, because p95 signal is too week to detect by Western blotting for phosphotyrosine, but the possibility exists that p95 might be related to p100 (i.e., the same protein with less phosphorylated tyrosine residues). It is known that tyrosine phosphorylation changes protein mobility in SDS-PAGE (Huang et al., 1995; Yan and Templeton, 1994).

The p100 and p95 proteins were separated on SDS-polyacrylamide gel, then cut out from the gel and electroeluted as described in Materials and Methods. Purification was confirmed with Western blotting and silver staining. The purified p95 was easily detected with anti-PY Western blotting.

4. Amino acid sequencing and possible future experiments

The p95 and p100 proteins have not been sequenced. Assuming sufficient sequence can be obtained, the next step

will be to search for proteins that have an identical or similar sequence with molecular size of p100 in protein databases. If we can not find such proteins in any databases, it would be more likely a novel protein, and we would try to identify mRNA of the protein in the SFME cells using a synthetic (degenerate) probe predicted from the amino acid sequence. If mRNA is successfully probed, the next step would be to clone the cDNA and sequence clones to determine the entire coding sequence of the gene.

Even in the absence of sequence, some information regarding the nature of p95 and p100 can be obtained from determination of the amino acid compositions. If the protein can not be sequenced due to the small amount, alternative approaches will be required to produce enough material for sequencing. We may make antibody by making hybridomas after immunizing mice by injection of the protein directly into mouse spleen in collaboration with investigators at the University of Oregon Monoclonal Antibody Facility. We could then purify p100 using antibody in immunoaffinity chromatography (with or without tyrosine phosphorylation) from Balb/c mice brain or other tissues. The antibody also could be used for more characterization of the protein, such as tissue specificity of expression and subcellular localization.

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