THE ROLE OF THE INSULIN-LIKE GROWTH FACTOR PATHWAY IN ETV6-NTRK3-MEDIATED TRANSFORMATION

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B.Sc. Biochem., The University of Victoria, 1993
MDCM, McGill University, 1997

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE O F

MASTER OF Science

In

THE FACULTY OF GRADUATE STUDIES

(Department of Surgery, University of British Columbia)

THE UNIVERSITY OF BRITISH COLUMBIA

OCTOBER 2001

Department of SURGERY

The University of British Columbia
Vancouver, Canada

Date November 9, 2001

ABSTRACT

Congenital fibrosarcoma (CFS) and cellular congenital mesoblastic nephroma (CMN) are pediatric spindle cell malignancies that are characterized by two specific cytogenetic abnormalities: trisomy of chromosome 11 and a t(12;15)(p13;q25) rearrangement. The t(12;15) translocation creates a transcriptionally active fusion gene that encodes a chimeric protein, ETV6-NTRK3 (EN). This fusion oncogene transforms NIH3T3 fibroblasts through constitutive activation of the Ras-MAP kinase (MAPK) pathway. In this study we demonstrate that trisomy 11 correlates with high expression of insulin-like growth factor-II (IGF-II) in CFS and CMN tumor samples using Northern blotting. We provide evidence that an intact IGF signaling axis is essential for EN-mediated transformation by expression of EN in IGF-I receptor (IGF-IR) knockout murine fibroblasts. In the absence of IGF-IR, EN is only partially transforming as measured by colony formation in soft agar. We have investigated the signaling pathways activated by IGF-IR and the EN fusion protein in NIH3T3 and IGF-IR knockout murine fibroblasts (R-cells) and we have found significant overlap and interdependence between these two pathways. Along with constitutive activation of the Ras-MAPK pathway, EN transformation is also dependent on constitutive activation of the phosphatidylinositol-3' kinase (PI-3K)-Akt survival pathway as demonstrated through the use of the specific PI-3K blocking agents, LY294002 and wortmannin, that revert transformation. The levels of activation of these key transformation pathways are significantly reduced in the absence of IGF-IR. In the presence and absence of IGF-IR, however, EN appears to interact with the major downstream substrate of IGF-IR, the insulin-receptor substrate-1 (IRS-1), as demonstrated by co-immunoprecipitation experiments. IRS-1 appears to be a substrate for the kinase domain of EN as it is constitutively

tyrosine-phosphorylated in the presence and absence of IGF-IR in EN expressing cells. Also, IRS-1 serves as a docking site for recruitment of Grb2 and p85 which are key elements involved in the activation of the PI-3K-Akt and Ras-MAPK pathways. Despite its ability to activate IRS-1, EN fails to confer a fully transformed phenotype to IGF-IR knockout cells. We speculate that IGF-IR may allow maximal activation of the PI-3K-Akt and MAPK pathways through direct binding and activation of upstream elements of these pathways independent of IRS-1 or that IGF-IR plays a role in translocating activated IRS-1 and EN to the inner cell membrane where they can act efficiently in a subcellular compartment enriched for upstream regulatory elements of the PI-3K-Akt and MAPK pathways.

TABLE OF CONTENTS

LIST OF FIGURE

page

CHAPTER 1

INTRODUCTION

1.1 Introduction to cancer

1.1.1 Cancer genetics

The defining features of cancerous cells relate to their ability to escape normal physiologic controls of cellular growth and spread to distant sites in the host. Cancer has been found to be fundamentally a genetic disease, and the genes involved in malignant progression produce proteins that function to dysregulate normal cellular processes such as mitosis, apoptosis and cellular growth. Mutation or aberrant expression of these genes allows the cancer cells to proliferate autonomously, avoid apoptosis, promote angiogenesis and metastasize to distant sites in the host.

Tumor suppressor genes, in a wild-type state, are involved in inhibiting cell growth. These genes become involved in promoting cancer when both alleles become mutated and inactivated. A classical example of a tumor suppressor is the p53 protein that regulates cell-cycle progression. In response to DNA damage, p53 expression prevents the cell from dividing so that repair of the affected nucleotides can be achieved. When both copies of p53 are mutated and rendered non-functional, the cell progresses through the cell-cycle despite DNA damage and as a result accumulates other mutations that lead to oncogenic progression (3).

Proto-oncogenes, on the other hand, act dominantly to promote growth and require mutation of only a single allele to render them oncogenic. Proto-oncogenes are usually involved in intercellular communication, signal transduction or regulation of

transcription. Under normal conditions the activity of these molecules is under tight control whereas when they become activated as oncogenes they are uncoupled from these upstream regulatory elements. The mutations involved in dysregulating these oncogenes vary from single point mutations to rearrangements and deletions of large portions of genes (80). An example of this is the *ras* proto-oncogene which is normally involved in promoting cellular proliferation and becomes activated when bound to GTP. A constitutively activated oncogenic version of *ras* results from a number of different mutations including point mutations that result in a single amino acid substitution that renders the protein unable to hydrolyze GTP (75).

Another example of proto-oncogene activation is the oncogene *v-erbB* which is the viral homologue of the epidermal growth factor (EGF) receptor protein tyrosine kinase. It becomes activated as a result of deletion of both a portion of the intracellular regulatory region and a portion of the extracellular ligand-binding domain. As a result, the *v-erbB* oncoprotein dimerizes and autophosphorylates in the absence of the ligand, resulting in constitutive activation of the EGF growth-factor signal transduction pathway. One of the major challenges facing cancer researchers is to identify the tumor suppressor and proto-oncogenes involved in specific malignancies and then characterize their functional roles.

1.1.2 Discovering cancer genes

In order to identify cancer causing genes a number of interesting approaches have been developed. Three main approaches have been used to identify specific genes involved in cancer progression.

One of the earliest techniques used to isolate cancer genes was developed from the study of animal tumor viruses. The fundamental understanding of cancer as a genetic disease dates back to work in which it was found that the transfer of specific sequences of DNA from animal viruses to cultured cells confers a cancerous or "transformed" phenotype. The study of these animal tumor viruses lead to the discovery and isolation of some the first oncogenes including the *v-src* oncogene of Rous sarcoma virus (63). c-*src*, the cellular homologue *v-src* has been further characterized as a membrane associated tyrosine kinase involved in growth and differentiation (89).

In the 1970's techniques were developed to fragment DNA from cancerous cells and transfer this DNA into non-cancerous cell lines and assay for transformation. This technique lead to the identification of such dominantly acting oncogenes such as *Ha-ras* (37). *Ha-ras* is a mutated version of r*as* which is a gene involved in stimulating the mitogen-activated protein-kinase pathway that is responsible for cell growth and differentiation. The *Ha-ras* oncogene contains a point mutation that results in decreased activity of the GTPase function of the enzyme and as a result the protein displays constitutive activation (24).

Another method for oncogene discovery has come about through the study of tumor cytogenetics. In the study of sarcomas and hematologic malignancies a number of recurrent non-random cytogenetic abnormalities have been found to be associated with certain malignancies. Examination of translocation breakpoints and amplified or deleted chromosomal regions has lead to the discovery of genes involved in cancer. Through the use of molecular cytogenetics, important oncogenes such as *c-myc,* a transcription factor, (34) and *c-abl,* a soluble tyrosine kinase, (72) have been discovered.

The identification of specific chimeric oncoproteins has also been realized through cloning of the precise breakpoints of translocation fusions. When a translocation occurs within the exons of two genes the resulting translocation may encode a chimeric oncoprotein (80). These chimeric oncoproteins result from the in-frame fusion of two genes resulting in the production of a novel protein composed of portions of each gene. One example of this is EWS-FLI1, a potent chimeric oncoprotein found in Ewing's sarcoma. This fusion is comprised of the EWS gene fused to FLI1, both of which are transcription factors (80).

Cytogenetics has also played an important role in identifying tumor suppressor genes involved in cancer. The initial work on tumor suppressors was done by Knudson (41) on retinoblastoma, an inherited form of childhood malignancy, where the age of distribution of the cases suggested the need for a "second hit" for malignancy to develop. Using cytogenetics, a deletion along chromosome 13 was detected. Deletion of this region along one chromosome was found to be inherited and further deletion of the corresponding region on opposite allele was found to be associated with tumor development. Using refined techniques the exact location of the deletion was mapped and found to involve the retinoblastoma gene which has subsequently been found to be involved in modulating cell-cycle regulation (99).

1.2 Growth factors in Cancer

Cellular transformation essentially results from a cell's acquired ability to proliferate uncontrollably and escape natural cell death mechanisms. Under normal physiological conditions these two very important cell processes are tightly regulated by

growth factor signaling pathways. The importance of growth factor pathways in tumorigenesis has been illustrated by the finding that the majority of dominant oncogenic mutations affect genes linked to these pathways. These oncogenic mutations lead to increased expression or constitutive ligand-independent activation of receptors or downstream intracellular elements of these pathways (13) (8)

Numerous mutations of protein tyrosine kinase growth factor receptors have been discovered as dominantly acting oncogenes. Many of these mutations have been extensively studied and the mechanisms by which they dysregulate physiologic function are now understood. Mutations in tyrosine-kinase receptors such as the epidermal growth-factor receptor, EGFR/ErbB1, alter the structure of the extracelluar ligand-binding sites have been found to allow ligand-independent kinase activity (13). Other tyrosine kinase receptors such as ErbB2 and IGF-IR have been found to be overexpressed in malignant tissues, leading to a greatly amplified response to endogenous levels of growth factors(62).

Other oncogenes have been found to promote transformation due to their ability to activate downstream components of growth factor pathways and in such a fashion induce increased growth signaling. An example of this mechanism is the polyoma virus middle T antigen which binds directly to Shc leading to activation of the Ras-MAPK signaling cascade (16).

1.3 Growth factor-related oncogenes in medicine

1.3.1 Improving diagnosis

The discovery of genes involved in cancer can have an immediate impact on clinical medicine. The presence or absence of specific oncogenes can be of diagnostic and prognostic importance. This has become the case with the discovery of ETV6-NTRK3, which serves to differentiate the more benign infantile fibromatosis from the histologically identical and more clinically aggressive tumors, congenital fibrosarcoma and cellular congenital mesoblastic nephroma (39). Another example is neuroblastoma, where the presence of amplified copies of the *N-myc* oncogene allows clinicians to identify a subset of disease with a more aggressive clinical course (82). It is hoped that, ultimately, a detailed understanding of specific active oncogenes will lead to the development of targeted therapy. This approach has become a reality in breast cancer where it has been discovered that a subset of carcinomas overexpress the Neu/ErbB2 receptor. Screening of breast cancer tumors for over-expression of Neu/ErbB2 not only improves diagnosis but also allows for a disease-specific treatment. A specific blocking antibody, Herceptin, has been developed to prevent signaling through this receptor and it is now used in clinical practice to treat this subset of breast cancer patients (97).

1.3.2 Designing treatment

Many of the current chemotherapeutic regimens rely on toxic, non-specific therapies that carry with them significant morbidity. In order to develop more bio-

specific treatment strategies a detailed understanding of the identity, structure and function of oncogenes is essential.

The identification of the genes involved in tumor formation can serve as an immediate template for the development of anti-cancer drugs. The knowledge of the nucleotide sequences of oncogenes can be used to develop antisense oligonucleotide therapies. Antisense oligonucleotides bind oncogene transcripts in a sequence-specific manner leading to degradation of mRNA and arrest of protein production (46).

Antisense oligonucleotides directed against IGF-IR mRNA have been developed and have demonstrated significant effects on down-regulating IGF-IR protein levels as well as on *in vitro* and *in vivo* tumor growth (2). These IGF-IR antisense oligonucleotides are now being used to treat malignant astrocytomas (2).

The study of cancer-causing genes at the protein level is also very important. The goal of this work has been to identify the intracellular signaling cascades dysregulated by specific oncoproteins as well as their direct binding partners. The 3-D structures of oncoproteins such as tyrosines kinases are studied with the goal of developing specific small molecule or dominant-negative inhibitors. By using x-ray crystallography, the 3-dimensional structure can be determined and used towards the rational design of small molecule inhibitors that block the active site of oncogenic kinases. Work with BCR-ABL is a successful example of this approach. BCR-ABL is a chimeric tyrosine kinase that was identified at the breakpoints of the t(9;22) Philadelphia chromosome found in chronic myelogenous leukemia. This chimeric tyrosine kinase is constitutively active as a result of homodimerization and autophosphorylation that is mediated by the BCR coiled-coil domain (50). Knowledge of the 3-D structure of this molecule was used to develop a

specific small molecule inhibitor, STI-571, that binds directly at the active site and prevents kinase activity. *In vitro* studies have demonstrated specific activity of STI 571 in BCR-ABL-transformed leukemic cells where it has been found to inhibit growth (36).

1.4 Congenital fibrosarcoma and congenital mesoblastic nephroma

1.4.1 Congenital fibrosarcoma

Congenital fibrosarcoma (CFS) is a spindle cell malignancy of infancy that is closely related histologically to congenital mesoblastic nephroma, infantile fibromatosis and adult type fibrosarcoma. CFS distinguishes itself in that it afflicts patients under the age of two years and manifests as a rapidly expanding lesion in the extremities. CFS is readily treatable with surgery and chemotherapy and the overall survival rate is 90% despite a high recurrence rate (40%) and metastatic rate of 10%(29).

1.4.2 Congenital mesoblastic nephroma

Congenital mesoblastic nephroma (CMN) is a closely related renal spindle cell malignancy that occurs predominantly in infants under the age of three months (14). It is composed of three subtypes: classical, mixed, and cellular, depending on the degree of cellularity (62). All three subtypes respond well to local surgical management, however there are reports of metastasis and recurrence found in the cellular variant (35)

1.4.3 Cytogenetic abnormalities found in CFS and CMN

Because of the histological similarity between CFS and CMN and many other malignancies there has been a focus on studying their cytogenetic profiles in order to

identify recurrent, non-random chromosomal abnormalities that may assist in pathologically differentiating these highly similar lesions. These studies have revealed similar chromosomal patterns in both CFS and cellular CMN. Virtually all cases of CFS have been found to contain a t(12;15)(p13;q25) rearrangement that encodes the fusion oncoprotein ETV6-NTRK3 (40, 73). As well, the majority of cases of cellular CMN have been found to contain the same t(12;15)(p13;q25) translocation.

Many of the CFS and CMN tumors have been found to have polysomies of chromosomes 8,17 and 20 whereas the majority have been found to have trisomies of chromosome 11 (76). Furthermore, there is a strong correlation between the presence of the trisomy of chromosome 11 and the t(12;15) rearrangement in cases of CFS and CMN (39, 73). More specifically, the majority of cases of classical CMN contain neither the t(12;15) nor the trisomy. In a recent study of 15 cases of CMN, 11 were found to contain the t(12;15) translocation and all of these cases contained trisomy 11 as determined by flourescent in-situ hybridization (FISH). There were no cases that contained either trisomy 11 or the t(12;15) alone (39).

The remarkable correlation between trisomy 11 and the presence of the t(12;15) associated ETV6-NTRK3 (EN) fusion suggests that trisomy 11 may play an important role in tumorigenesis. One gene located on chromosome 11 that has been implicated in other pediatric malignancies is the insulin-like growth factor-II (IGF-II) gene (92). This gene encodes a peptide growth factor that has been found to be expressed at high levels in other pediatric malignancies such as neuroblastoma, rhabdomyosarcoma and Wilm's tumor (85). There is evidence to suggest that it plays a role in preventing apoptosis and

thus allows cells to accumulate mutations and progress towards a malignant phenotype (19).

1.5 ETV6-NTRK3 fusion oncoprotein

1.5.1 ETV6-NTRK3 molecular structure

The EN fusion oncoprotein results from the in-frame fusion of the first five exons of the ETV6 gene, a member of the ETS family of transcription factors, with exons 13-18 of the NTRK3 neurotrophin receptor tyrosine kinase gene (40). As a result, the helix-loop-helix (HLH) dimerization domain of the ETV6 gene is fused to the cytoplasmic tyrosine kinase domain of NTRK3.

The wild-type ETV6 protein is composed of an N-terminal HLH domain that allows dimerization and a DNA-binding domain which specifies promoter binding. This molecule serves to regulate transcription of genes by binding specific promoters and activating or suppressing gene expression. It is felt to play an important role in early angiogenesis or hematopoesis (92). Interestingly, ETV6 has been found to be associated with a number of leukemia-associated translocations including fusions with: PDGFRβ (33), JAK2 (65) and ABL (60).

The NTRK3 portion of the fusion consists of the intracellular domain of a transmembrane growth factor receptor of the Trk family of nerve growth factor receptor tyrosine kinases. Other members of the Trk family include NTRK1, NTRK2 and the p75 neurotrophin receptor (68). The Trk family of receptors bind the neurotrophins which are required for neuronal proliferation, differentiation and survival (6). Upon ligand binding the receptors undergo dimerization and autophosphorylation. Activation of NTRK3 by

binding of the NT-3 ligand and subsequent autophosphorylation leads to stimulation of downstream signaling pathways.

1.5.2 Oncogenic properties of ETV6-NTRK3

The oncogenic properties of the fusion protein were initially demonstrated by expressing EN in NIH3T3 fibroblasts. The EN-transfected NIH3T3 fibroblasts were rendered transformed morphologically and were found to form colonies in soft agar and tumors in nude mice (91). The ability of the fusion protein to homodimerize through the HLH domain was demonstrated *in vitro* and mutants that lacked the HLH domain were found to be non-transforming when expressed in 3T3 fibroblasts. The kinase domain of the NTRK3 portion was found to be essential for transformation as a mutant of the ATP-binding site was found to be non-transforming (91). The conclusions from this work are that the fusion requires dimerization and autophosphorylation in order to mediate dysregulated growth-factor signaling that leads to transformation.

1.5.3 NTRK3 signal transduction

The NTRK3 receptor is activated by binding of the NT-3 ligand which leads to autophosphorylation of the receptor on the intracellular tyrosine residues: 516, 705, 709, 710 and 820 (6, 38). A number of adapter proteins have been linked to NTRK3 activation of downstream signaling cascades. At tyrosine 516 in the juxtamembrane region of NTRK3 the adapter proteins Shc and p85, the regulatory subunit of PI-3K, have been demonstrated to bind via their src-homology-2 (SH2) domains (58). Phospholipase-Cγ has been found to bind to the C-terminal portion at tyrosine 820 (45). Other adapters that

have been found to bind NTRK3 and activate the MAPK pathways include FRS-2 (42), rAPS (51), SH2-B (68) and CHK (96).

The Shc adapter protein recruits growth factor-bound protein 2 (Grb2) which in turn binds the Ras guanine exchange factor, Son-of-sevenless (Sos), to activate the MAPK pathway (23). The binding of the p85 regulatory subunit of PI-3K leads to recruitment of the p110 catalytic subunit of PI-3K. The active PI-3K holoenzyme phosphorylates lipid substrates to generate phosphatidylinositol-3,4,5-trisphosphate which recruits the serine/threonine kinase, Akt, to the membrane(23). Akt becomes activated by phosphorylation at two sites, serine 473 and threonine 308, by phosphoinositide-dependent kinase 1(PDK1)(22).

Akt activation is involved in promoting cell survival (24). It is not entirely clear by which mechanisms Akt promotes cell survival but current evidence indicates that Akt acts at a number of different levels to prevent induction of apoptosis. Apoptosis, or programmed cell death, is a multistage process that involves, as an early event, the loss of mitochondrial cell membrane integrity with the release of cytochrome C. Cytochrome C binds to the apoptotic protease-activating factor (Apaf-1) which leads to the cleavage and activation of caspase 9 (83).

Akt has been found to inactivate pro-apoptotic Bcl-2 family proteins such as Bad, which, in the absence of Akt phosphorylation, forms a heterodimer with the anti-apoptotic protein Bcl-2. Phosphorylation of Bad at serine 136 causes it to release the anti-apoptotic protein Bcl-2 (20). The importance of Bad as a major substrate is unclear as it is expressed at very low levels in tissues and is not ubiquitously expressed. Akt is

also thought to exert anti-apoptotic action via its ability to phosphorylate and inactivate GSK3 (59), caspase 9 (81) and the Forkhead transcriptional factors (15).

Activation of phospholipase Cγ (PLCγ) by phosphorylation leads to PLCγ hydrolysis of phosphatidylinositol bisphosphate to generate the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is involved in Ca^{2+} release whereas DAG is required for the activation of protein kinase C (12).

1.5.4 ETV6-NTRK3 signal transduction

The CFS t(12;15) rearrangement splices the first five exons of ETV6 with exons 13-18 in NTRK3. As a result, the NTRK3 portion of the fusion contains the tyrosine kinase domain but not the juxta-membrane tyrosine 516 that has been demonstrated to bind Shc and p85. In recent work it has been demonstrated that the CFS EN fusion does not bind p85, Shc or Grb2 (91). As well, it has been demonstrated that mutation of tyrosine 820, the PLCγ-binding site, does not affect the transforming potential of the fusion (91).

The EN fusion, however, has been found to constitutively activate the Ras-MAPK pathway as is demonstrated by constitutive activation of Mek1/2 and constitutive expression of cyclin D1 (C.Tognon, Cancer Reseach ,in press). Use of dominant negative forms of Ras and the Mek1/2 inhibitor, U0126, leads to partial and complete abrogation of transformation in the NIH3T3 fibroblast model system. This experimental evidence underlines the essential nature of this pathway in EN-induced transformation.

Although it is clear that EN induces transformation via activation of the Ras-MAPK pathway it is unclear how it initiates activation of these pathways as all major upstream adapter proteins investigated do not appear to interact directly with EN.

1.6 Insulin-like growth factor (IGF) axis

1.6.1 Composition of the IGF axis

The presence of trisomy 11 in EN-expressing CFS and CMN tumors has stimulated interest in investigating the expression level of the insulin-like growth factor-II (IGF-II) gene which is located in the 11p15.5 region on chromosome 11. IGF-II is a peptide mitogen that interacts with the insulin-like growth factor I receptor (IGF-IR). Stimulation of the IGF-IR leads to the activation of a set of pathways involved in important cellular process such as mitosis, cell survival, differentiation and transformation (56). The importance of the IGF pathway in growth and development has been illustrated from the phenotype of the IGF-IR knockout mouse. Knockout mice which are null for the IGF-IR were stillborn and found to be only 45% the size of normal mice (4).

The IGF axis is comprised of two specific ligands, IGF-I and IGF-II, along with six specific IGF binding proteins and two receptors, the IGF-I and IGF-II receptors (9). As well, insulin is able to interact with and stimulate the IGF-IR (9). IGF-I and IGF-II are small peptide mitogens of approximately 7.5-15 kDa in size that have 70% overall sequence homology. IGF-I binds with the highest affinity to the IGF-IR whereas IGF-II and insulin bind with 10-fold and 100-fold lower affinity respectively. IGF-II also binds to the IGF-II receptor that is thought to function by sequestering and degrading IGF-II

(53). The IGF's are ubiquitously expressed and act in either an autocrine, paracrine or endocrine manner. They circulate in the blood in association with the specific IGF binding proteins which act to either enhance or suppress their interactions with the IGF-IR (57).

1.6.2 IGF axis in malignancy

Dysregulation of the IGF axis has been discovered in a number of adult and pediatric malignancies. In pediatric malignancies the IGF-IR has been found to be upregulated in Wilm's tumor (30) and neuroblastoma (25, 27). The IGF-II ligand has been found to be overexpressed in rhabdomyosarcoma (26) as well as in Wilm's tumor and neuroblastoma.

The functional role of an overactive IGF axis in malignancy appears to be largely related to its ability to suppress apoptosis. In a number of different *in vitro* models the ability of IGF to suppress apoptosis is critical for transformation. A set of experiments in Rat-1 fibroblasts expressing *c-myc* have shown IGF-I to be an extremely potent survival factor for these cells in low serum (28). In an animal model expressing SV40 large T-antigen specifically in the pancreas it was found that IGF-II expression was required in order for hyperplastic lesions to progress to tumors (19).

The IGF-IR itself appears to play a very important role in cellular transformation as has been outlined by a number of interesting *in vitro* experiments. Much of this work has focussed on working with murine IGF-IR knockout fibroblasts: the "R-" cells. It has been found that a number of very potent oncogenes such as *Ha-Ras*, EWS-FLI-1 and SV40 large T antigen are unable to render a transformed phenotype in these knockout

cells (9). The importance of IGF-IR in tumorigenesis has also been discovered in various animal models of cancer.

A number of different strategies have been used to down-regulate or inhibit IGF-IR. A specific monoclonal antibody directed against IGF-IR has been used to inhibit tumor formation by breast cancer cell lines in nude mice (57). Peptide analogues directed to block IGF-IR have been used successfully to inhibit the growth of prostatic cell lines (67). Antisense oligonucleotides have also been used successfully directed against IGF-IR to inhibit the growth of rat glioblastoma cells, human melanoma cells and mouse leukemia cells (70, 71).

1.7 Thesis Objectives

The translocation-associated fusion oncoprotein, ETV6-NTRK3, found in CFS and CMN is correlated with trisomy 11 in virtually all cases investigated (39). Trisomy 11 has been found to be associated with increased levels of IGF-II transcripts by other research groups. We believe that there may be a functionally important link between trisomy of chromosome 11 and the presence of the EN fusion protein in CFS and CMN tumors. More specifically, we hypothesize that the IGF system may serve an essential function in ETV6-NTRK3-mediated transformation. In order to investigate this hypothesis we established a number of specific experimental objectives.

1. To test whether IGF II is overexpressed in CFS or CMN.
2. To express the fusion oncoprotein ETV6-NTRK3 in murine fibroblasts that lack IGF-IR (R-) and undertake experiments to determine if these cells are transformed.

3. To characterize other EN-activated pathways aside from the Ras-MAPK pathway that are important or essential for EN transformation in NIH3T3 cells.
4. To determine whether specific components of the IGF-IR axis are involved in activation of EN-associated transformation pathways.

CHAPTER 2

MATERIALS AND METHODS

2.1 Tissue culture

IGF-I receptor knockout mouse embryo fibroblasts (R- cells) and P6 cells, a fibroblast cell line engineered to overexpress IGF-IR, were kind gifts from Dr. Renato Baserga (Kimmel Cancer Centre, Philadelphia). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM Life Technologies) with 9% fetal bovine serum. R- cells were maintained in media containing 34mg G418/litre (Sigma). Cells were cultured at 37°C with 5% carbon dioxide. NIH3T3 cells were obtained from the American Type Culture Collection and were cultured in DMEM high glucose, with 9% calf serum. BOSC 23 packaging cells, used to produce ecotropic viruses, were cultured in 9% fetal bovine serum in DMEM. BOSC 23 cells were a kind gift from Dr. Rob Kay (Terry Fox Laboratories, Vancouver).

2.2 Transfections and vectors

Transfections of R- and 3T3 fibroblasts were carried out using the Murine Stem Cell Virus (MSCV) Retroviral Expression System from Clontech. Briefly, the BOSC23 packaging cell line was transfected with target plasmids using the calcium phosphate method as previously described (64). Creation of R-EN and 3T3 EN cell lines was achieved with the use of MSCV puromycin vectors containing the ETV6-NTRK3 cDNA (accession # AF041811). The R+ cell line was created using the MSCV hygromycin vector containing the full length IGF-IR cDNA which was created in our lab as follows. A SalI/EcoRI fragment of pGR13 (Dr. Renato Baserga) containing the full length cDNA

for IGF-IR was ligated into the XhoI/EcoRI sites on MSCV hygromycin. Verification of the identity and orientation of the IGF-IR cDNA in MSCV hygromycin was carried out using restriction enzyme digest analysis. Cells expressing activated *Ha-ras* were created by transfection of pCTV 2.11 containing activated V12 *Ha-ras* (gift from Dr.Rob Kay Terry Fox Laboratories, Vancouver) in conjunction with the BOSC 23 packaging system and puromycin selection (91).

2.3 Northern blotting

Total RNA was extracted from primary frozen tumor samples and cell lines using acid guanidinium-phenol/chloroform method as previously described (74). Northern blotting was carried out using 20 micrograms of total RNA using standard protocols (74). A 500 base-pair IGF-II probe was generated from CMN cDNA using primers specific for exon 9: sense primer: 129 5'-CTTGGATTTGAGTCAAATTGG-3' and antisense primer: 131 5'CCTCCTTTGGTCTTACTGGG-3'. The ß-actin probe was obtained from Clontech (cat # 9800-1).

2.4 Soft agar assays

Cells were plated at a density of 2,000 cells per milliliter of 0.2% agarose supplemented with 10% FBS over an underlayer of 0.4% agarose/10% FBS in DMEM. Each experiment was carried out in triplicate in 35mm wells for each cell line. Each cell line was examined in a minimum of seven separate soft agar experiments. Colony formation was assessed at two weeks as macroscopic colony formation. All colonies greater than 0.1 mm in size were counted along with all single cells in each high powered

field (40X objective). Ten high powered-fields per well were counted for a total of thirty counts per experiment. The results are represented as a total number of colonies per total cells (single cells plus colonies). Each experiment was carried out a minimum of eight separate times. Statistical analysis was carried out using a paired student's t-test.

2.5 Western blotting and immunopreciptation

Prior to lysis, cells were cultured in either 9% serum or in 0.5% serum for 18hrs (to synchronize in G_0) and then stimulated with 9% serum or differing concentrations of purified IGF-I or IGF-II as indicated in the figure legends. Cells were cultured to ~75% confluence, and either starved or stimulated as indicated and then rinsed twice with PBS followed by lysis as previous described (91). Cell lysates were cleared by centrifugation and protein concentrations were determined using Biorad protein quantification kit (Dc Protein Assay kit). Immunoprecipation was carried out with protein A-conjugated sepharose beads (Amersham) overnight at 4°C with mixing. The beads were washed three times with lysis buffer. Samples for both Western blotting and from immunoprecipitation were mixed with Laemmmli buffer and separated by electrophoresis on 10-15% gels according to standard protocols (74). The proteins were transferred to Immobilon-P membrane (Millipore) and blocked in 5% bovine serum albumin. Analysis was carried out with the indicated antibodies and detection was achieved with ECL according to the manufacture's protocol (Amersham). The antibodies used were as follows: α-IR3 α-IGF-IR mouse monoclonal (Calbiochem), α-Grb2 mouse polyclonal (Transduction), α-phospho-Mek1/2 Ser217/221 rabbit polyclonal (New England Biolabs,(NEB)), α-phospho-Akt Ser473 rabbit polyclonal (NEB), α-Cyclin D1/2 mouse

monoclonal (Upstate Biotechnology), α-IGF-IR β-subunit rabbit polyclonal antibody (Santa Cruz), α-NTRK3 rabbit polyclonal antibody (Santa Cruz) and anti-C-terminal IRS-1 rabbit polyclonal (Upstate Biotechnology.), α-p85 PI-3K (Transduction Laboratories), α-PLCγ (Transduction Laboratories), α-Tel rabbit polyclonal (P.Marynen). Purified IGF-I and II peptides were obtained form Sigma.

2.6 Site-directed mutagenesis

Site-directed mutagenesis was carried out using the Stratagene QuikChange Site Directed Mutagenesis Kit. This was used to create the EN PLCγ-binding as well as the kinase dead (EN-K380N) mutants using methods as previously described (91). The Y560F and Y594F were created using the same kit using the following primers:5'-GAGATCTTCACCTTTGGAAAGCAGCC-3' for the Y560F mutant, and the 5'-CCCAAAGAGGTGTTCGATGTCATGCTG-3' for the Y594F mutant. The precise DNA sequence of the mutants created was verified by sequence analysis. The PCR cycling parameters were as follows: one 30 seconds denaturation at 95°C, then 18 cycles of 95°C for 30 seconds, 55°C for one minute, 72°C for one minute followed by a final incubation of 68°C for 10minutes.

CHAPTER 3

RESULTS

3.1 IGF-II is overexpressed in CFS and CMN tumor samples

A strong correlation has been established between the expression of EN and trisomy 11 in cases of cellular CMN and CFS (11). We suspected that a trisomy of chromosome 11 may correlate with overexpression of IGF-II in CMN and CFS. To address this question, we characterized IGF-II mRNA expression from primary tumor sample specimens of cellular CMN, classical CMN, CFS and infantile fibromatosis, a closely related benign spindle cell tumor of infancy. We extracted total RNA from these samples and performed Northern blotting (Figure 1).

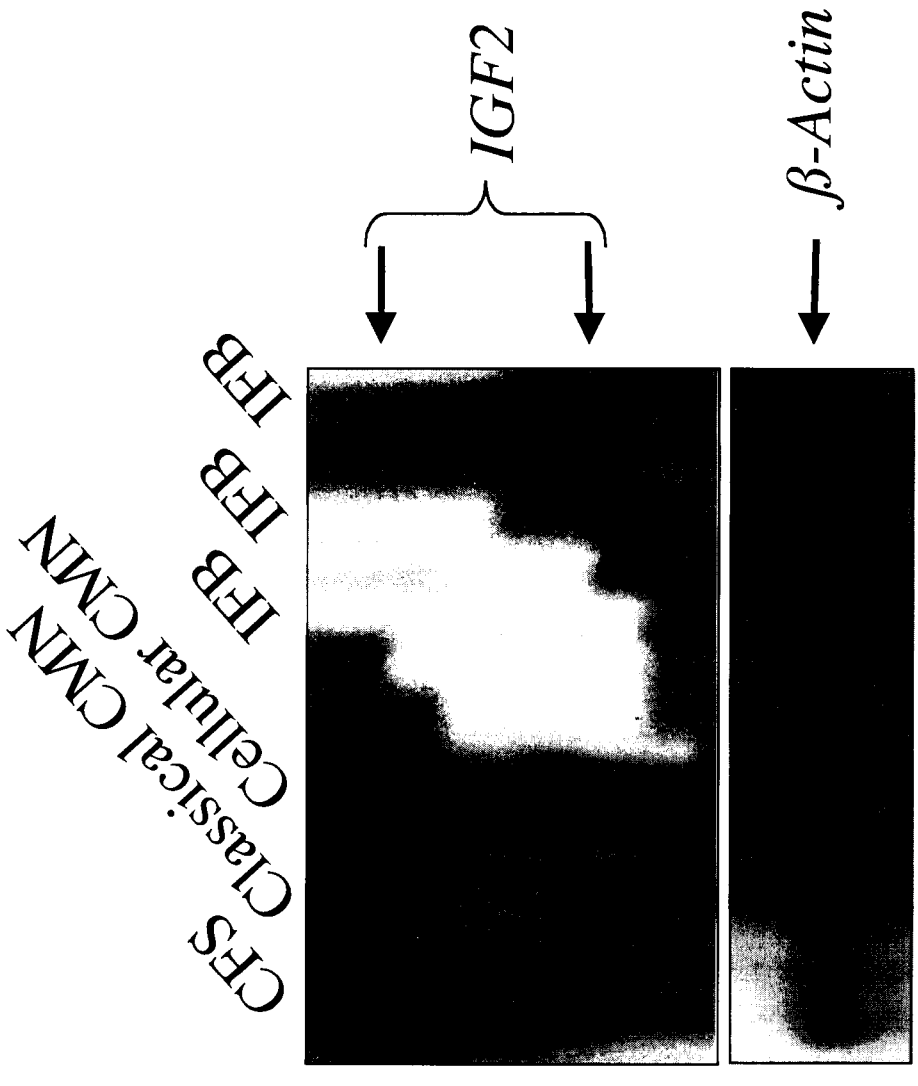
Northern blotting demonstrates high expression of IGF-II transcripts in one case of cellular CMN and one case of CFS with lower levels in a case of classical CMN. The levels of IGF-II message in the cases of IFB are negligible (Figure 1). Competitive RT PCR was used to confirm these results in five cases of CMN and CFS and this demonstrated between 10-30-fold higher expression of IGF-II transcripts in the tumor samples as compared to normal fibroblast controls (M-H Iglesias and C.Deal, Montreal, unpublished data, data not shown). These results are consistent with other reports of IGF-II overexpression in CMN (79).

3.2 Transfection of ETV6-NTRK3 (EN) into R- IGF-IR knockout murine fibroblasts

Given the high expression of IGF-II in EN-expressing tumors we postulated that the IGF axis may serve an important function in EN-mediated transformation. Using the

Figure 1.

IGF-II transcripts are overexpressed in CFS and cellular CMN. Total RNA was extracted from primary tumor samples and used for Northern blotting. A relative over-abundance of IGF-II transcripts is demonstrated in CFS and cellular CMN cases as compared to cases of infantile fibromatosis (IFB), a histologically similar tumor. These results were confirmed by competitive RT PCR in four other cases of cellular CMN and CFS (M-H Iglesias and C.L. Deal unpublished data, data not shown).


IGF2
β-Actin
CFS
Classical CMN
Cellular CMN
IFB
IFB
IFB

MSCV retroviral system and the BOSC 23 packaging cell line, infectious virus encoding EN, IGF-IR, and *Ha-ras* were generated and used to create the following cell lines: R-MSCV, R-EN, R-Ha-Ras, R+ (R-IGF-IR), R+EN and R+Ha-Ras. The R-Ha-Ras line was generated as a negative control as previous work has revealed this line to be only partially transformed as it forms foci in monolayer culture but generates negligible numbers of colonies in soft agar (31). The R+Ha-Ras was generated as a positive control for the soft agar and foci formation assays. The R-MSCV line and the R+ lines were also generated to function as controls.

In order to confirm protein expression of IGF-IR and EN constructs in the R- cells, Western blotting was performed (Figure 2). Figure 2 demonstrates the presence of the characteristic 68 kDa and 73 kDa doublet of EN present in the R+EN and R-EN cell lines. The EN doublet is due to the alterative ATG start site in the ETV6 portion of EN.

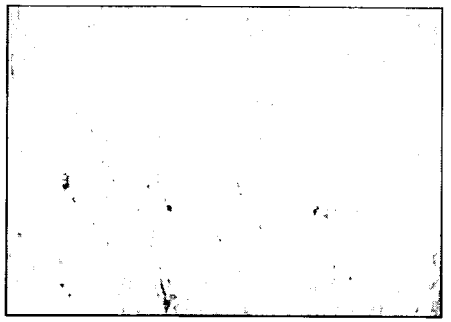
The presence of IGF-IR in the transfected cells is demonstrated by immunoprecipitating IGF-IR with α-IR3 α-IGF-IR monoclonal antibody and then probing with an α-IGF-IR β-subunit antibody. Figure 2 demonstrates the 97 kDa β-subunit present in the R+ cells and in a lysate from a P6 cell line that is engineered to overexpress IGF-IR (positive control).

Photographs of the R-MSCV, R-EN and R+ EN cell lines are represented in Figure 2. The vector control R-MSCV line retains the characteristic features of a non-transformed cell line, demonstrating a flattened appearance with a characteristic contact inhibited phenotype. The R-EN cells are more spindle shaped and demonstrate loss of contact inhibition. The R+EN cells are more compact and spindle-shaped with marked loss of contact inhibition. These features are consistent with a transformed phenotype.

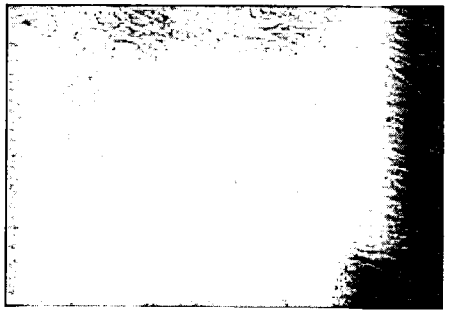
Figure 2.

Expression of ETV6-NTRK3 (EN) and re-expression of IGF-IR in R- cells. (A) R- cells transfected with MSCV empty vector display a flattened, contact-inhibited, non-transformed phenotype, R-EN cells are more spindle shaped whereas R+EN cells display complete loss of contact inhibition and form foci in monolayer culture. (B) Immunoprecipitation of cell lysates from R-EN, R-H-Ras and R-MSCV (Vector) cells with α-HLH Tel antibody and subsequent Western blotting with α-Trk C antibodies demonstrates the presence of the 68 kDa and 73 kDa doublet of EN. (C) The IGF-IR was re-expressed in R- cells by retroviral transfection and expression was verified by immunoprecipitation with α-IR3 monoclonal and Western blotting with α-β-subunit IGF-IR antibody (arrow indicates 97 kDA β-subunit). P6 cell line serves as a positive control.

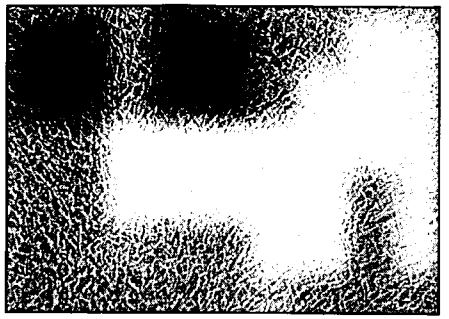
A.



R-

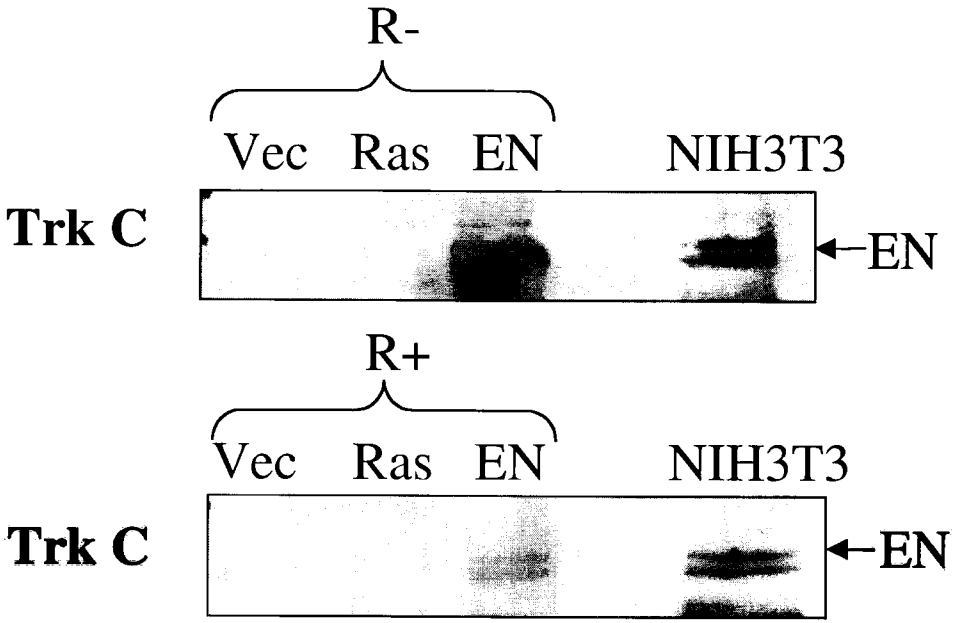


R-EN

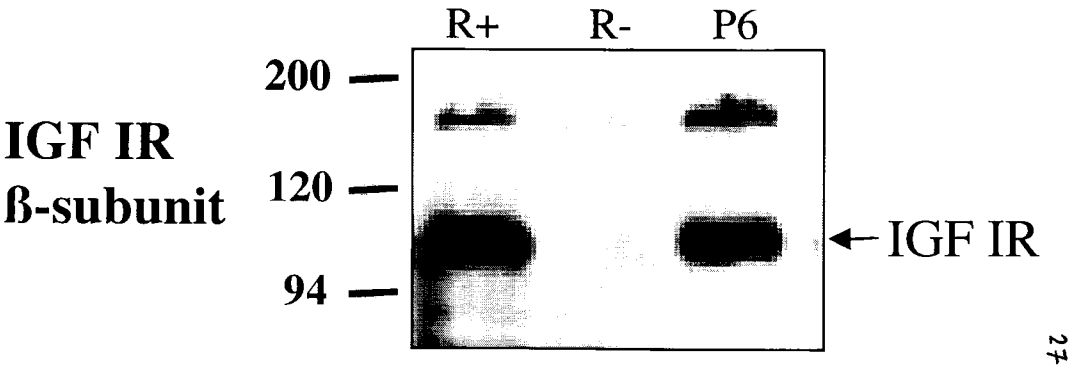


R+EN

B. IP: Tel



C. IP:IGF IR



3.3 EN-expressing R- cells show reduced soft agar colony formation

In order to assess the role of IGF-IR in EN-mediated transformation we looked at the ability of R-EN and R+EN cells to form foci in monolayer and form colonies in soft agar. The generally accepted criteria for assessing transformation outlined by Ponten describes three stages of transformation (77). Firstly, as cells progress to a transformed phenotype they demonstrate lower requirements for growth factors and increased saturation density. Next, they demonstrate loss of contact inhibition and form foci in monolayer (partial transformation) and finally, transformed cells are able to grow in an anchorage-independent manner and form colonies in soft agar and tumors in nude mice (full transformation) (88).

We undertook experiments to characterize focus formation in monolayer and colony formation in soft agar. The R- MSCV cells retain a contact-inhibited phenotype and fail to form foci in monolayer whereas R-Ha-Ras, R-EN, R+, R+EN and R+Ha-Ras display increased saturation density and foci formation in monolayer (data not shown). These findings are consistent with previous work by Sell and Baserga (77) who found that *Ha-ras* transfected R- cells are partially transformed.

To assess the capacity for growth in anchorage-independence, cells were plated in soft agar. Figure 3 shows a representative plate of the R-EN and R+EN cell lines after two weeks of growth. Colonies larger than 0.1 mm along with all single cells were counted in ten high-powered fields per well and the results in Figure 4 are a summary of these experiments. The R-Ha-Ras and R-MSCV cell lines demonstrate very low frequencies of colony formation with ratios of 0.03 and 0.008 colonies/ total cells (c/tc)

respectively. The R+ cell line generated in our lab, shows an intermediate level of colony formation which is statistically significantly different than the levels found in R+EN cells (p-value=0.00030931) and the levels in R-Ha-Ras and R-MSCV (p-value= 0.02215262 and p= 0.01884167 respectively).

The rate of colony formation for the R- MSCV cell line demonstrates very low numbers of colonies with an observed rate of 0.008 colonies/total cells (c/tc). R+Ha-Ras cell line demonstrates a high rate of colony formation of 0.4 c/tc. The R-EN cell line proves to have a significantly lower level of colony formation, 0.078 c/tc, as compared with R+EN, 0.38 c/tc (p-value=0.00011958), and R+Ha-Ras which are the positive controls. This rate of colony formation for R-EN is not, however, significantly different from the rate of colony formation in the R-Ha-Ras (p-value= 0.16452948) and R-MSCV lines, the negative controls. These results provide evidence that the R-EN is only partially transformed because they demonstrate loss of contact inhibition in monolayer but do not form significant numbers of colonies in soft agar. We conclude that IGF-IR is required for full EN-mediated transformation.

3.4 ETV6-NTRK3 transformation pathways

Previous work by C.Tognon has demonstrated that EN induces transformation through constitutive activation of the Ras-MAPK pathway (C.Tognon, Cancer Research, in press) as well as via upregulation of cyclin D1. This pathway has been found to be essential for transformation, as blocking of this pathway with dominant negative Ras and the Mek1/2 inhibitor, U0126, causes complete reversion of the transformed phenotype.

Another important pathway that has been found to be implicated in transformation by many oncoproteins is the phosphatidylinositol 3' kinase-serine/threonine protein

Figure 3.

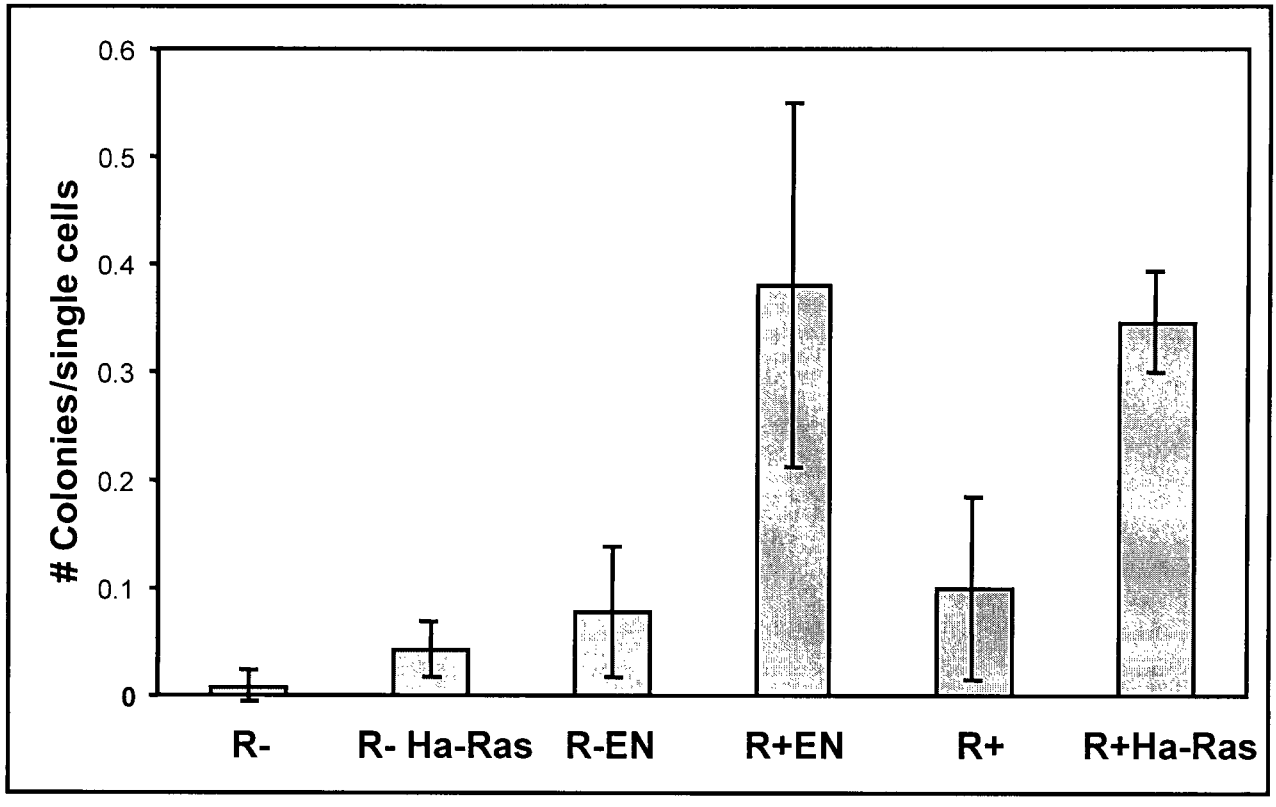
Soft agar plates of R-EN and R+EN. A photograph of a soft agar 6-well plate demonstrating high levels of colony formation in R+EN (lower three wells) compared to R-EN (upper three wells) is illustrated. Cells were plated at a density of 2,000 cells per mL of media in 0.2% agarose and 10% FBS over 1.5mL of underlay composed of 0.4% agarose with 10% FBS.

R-EN

R+EN

Figure 4.

Colony formation in soft agar. Cells were seeded at a density of 2 000 cells/mL of 0.2% agarose media as described in materials and methods. Results are presented as a summary of eight separate experiments. Colony formation is represented as a ratio of colonies to single cells. R+EN shows a statistically significant higher rate of colony formation as compared to R-EN and and R+ cell lines (p-value <0.05 using the student t-test). R-Ha-Ras forms negligible levels of colonies as previously described which is not statistically different from R-EN (77).



Cell line	# Colonies/total cells
R-	0.008
R- Ha-Ras	0.043
R-EN	0.078 ✷
R+	0.10 †
R+EN	0.38125 ✷†
R+Ha-Ras	0.3456

† p-value <0.01

✷ p-value <0.01

kinase Akt (PI-3K-Akt) pathway (24). Given the established role of NTRK signaling in activating the PI-3K-Akt survival pathway (68), we carried out experiments to determine if this pathway is involved in EN transformation. In order to determine if this pathway is involved in transformation we cultured NIH3T3 cells +/- EN as outlined in Figure 5 legend.

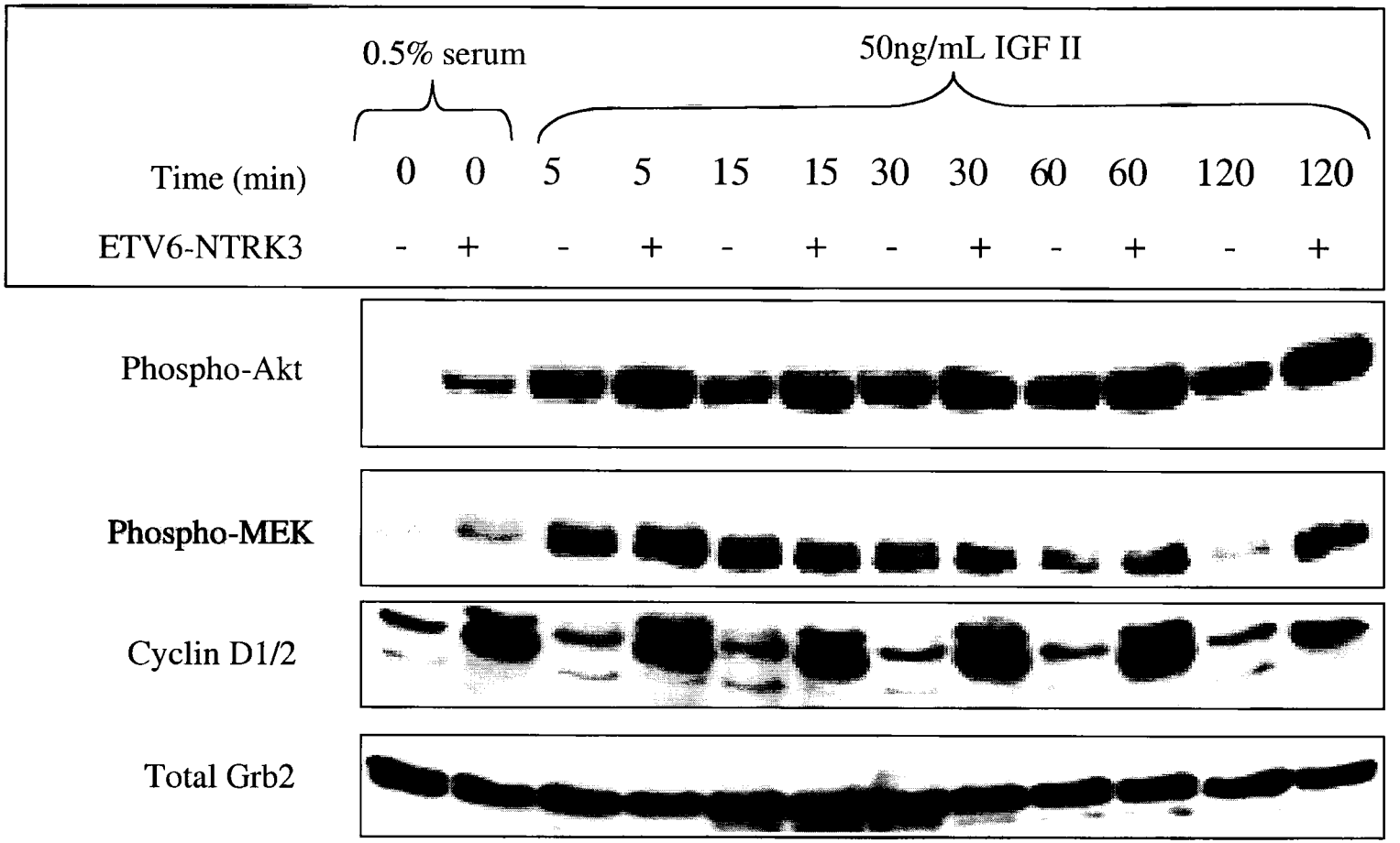
As previously demonstrated, we found that the presence of EN causes constitutive activation of Mek1/2 and upregulation of cyclin D1. However, we also found that EN expression is associated with constitutive activation of Akt as we demonstrated the constitutive phosphorylation at Akt Ser473 even in the absence of serum (left side, Figure 5).

3.5 Analysis of the PI-3K-Akt pathway in EN transformation

To assess the role of the PI-3K-Akt pathway in transformation, cells were treated with the specific PI-3K blocking reagents, LY294002 and Wortmannin, in order to block Akt activation. In monolayer it was found that treatment of cells with the PI-3K inhibitor, LY294002, has no effect on the morphology of EN-expressing 3T3 fibroblasts (data not shown). We next carried out soft agar experiments to determine the ability of PI-3K blockade to prevent formation of soft agar colonies. The cells were plated in 0.2% soft agar as described above in the presence of LY294002 25μM and wortmannin at 1nM and 2nM. We noted a dramatic decrease in the number of soft agar colonies in the presence of the PI-3K inhibitors. In the absence of PI-3K inhibitors EN+ NIH3T3 cells produce 0.42 colonies/total cells (c/tc) whereas in the presence of 1nM wortmannin there are 0.12 c/tc

Figure 5.

ETV6-NTRK3 signaling in NIH3T3 cells with IGF stimulation. EN-expressing NIH3T3 cells demonstrate constitutive baseline activation of Akt and Mek1/2 as indicated by increased phosphorylation of Akt Ser473 and Ser217/221 of Mek1/2 in serum deprived samples. With IGF-II stimulation there is a rapid and sustained (over 2hrs) activation of Akt in the NIH3T3 vector-transfected cells. Activation of Mek1/2 follows IGF-II stimulation in the NIH3T3 vector- transfected cells however returns to baseline at two hours post-stimulation. Cells were grown in 35mm dishes to 75% confluence and then serum-deprived in 0.5% serum DMEM for 18hrs after which they were stimulated with 50ng/mL IGF-II for the indicated times.


0.5% serum
50ng/mL IGF II
Time (min)
0 0 5 5 15 15 30 30 60 60 120 120
ETV6-NTRK3
- + - + - + - + - + - +
Phospho-Akt
Phospho-MEK
Cyclin D1/2
Total Grb2

and in the presence of 2nM wortmannin and LY294002 there are 0.02 c/tc and 0.0 c/tc respectively (see Figure 6).

In order to ensure that the LY294002 effect specifically blocks Akt activation we carried out Western blotting on cells treated with the inhibitors. We found that LY294002 treatment induced virtually a 100% block of Akt phosphorylation of Ser473. In contrast we found that the levels of activated Mek1/2 remain elevated in the EN-expressing cells and are essential unaffected by the PI-3K inhibitors (Figure 7).

3.6 IGF-IR activation causes sustained activation of Akt and transient Mek1/2 activation

The soft agar and monolayer morphology data suggest that activation of the IGF-IR pathway is essential for EN-mediated transformation. In order to determine if the IGF pathway overlaps and enhances the EN-induced transformation pathways or if it acts in an independent manner we wanted to see if the Ras-MAPK and the PI-3K-Akt pathways were affected by IGF stimulation in our 3T3 model system. The results of these experiments can be seen on the right hand side of Figure 5.

The addition of IGF-II increases activation of Mek1/2 in both the control cells and the EN-expressing cells. The effect in the control cells is transient and the activated Mek1/2 levels return to baseline at two hours. There is no effect of IGF-II stimulation on the levels of cyclin D1. We note that with the addition of IGF-II, the levels of activated Akt increase in both the control cells and in the EN-expressing cells. This work suggests that IGF-IR activation appears to enhance constitutive activation of Akt and Mek1/2 by EN.

Figure 6.

PI-3K inhibitors block colony formation in soft agar. NIH3T3 +/-EN cells were plated in soft agar containing either wortmannin (1nM or 2nM) or LY294002 (25μM) and assayed for their ability to form colonies. All colonies greater than 100 μM were counted at fourteen days. Each treatment was performed in triplicate and each experiment was performed 3 times. Results are presented as a percentage of colonies formed per number of total cells plated in each case.


% of Colonies Formed/#of Cells Plated
50%
40%
30%
20%
10%
0%
MSCV
ETV6-NTRK3
Wortmannin (1nM)
Wortmannin (2nM)
LY294002 (25μM)
+
+
+
+
+
+
+
+
+
+
+
+
+
+
+

These results confirm previous work that report that IGF has a well described role in activating the Ras-MAPK and PI-3K-Akt pathways in fibroblasts (9). The IGF-IR binds both Shc and IRS-1, its major substrate, to activate these two pathways. This work demonstrates that EN is able to activate both of these pathways in the absence of serum and that stimulation with IGF-II enhances signaling through these pathways.

It therefore appears that there is significant overlap between the IGF-IR pathway and the EN-mediated transformation pathway. Next, we carried out experiments to examine the relative importance of IGF-IR in contributing to activation of Akt and Mek1/2 in this murine fibroblast model system.

3.7 Akt and Mek1/2 activation is attenuated in the absence of IGF-IR

Given the critical nature of the PI-3K-Akt and Ras-MAPK pathways in EN transformation we hypothesized that the low grade transformation of the R- cells (by EN) could be due to reduced or absent activation of Mek1/2 and/or Akt. We therefore examined the activation of these proteins in the R- system.

Under low serum conditions the R-EN and R+EN cells demonstrate a similar level of basal activation of both Mek1/2 and Akt which is greater than that observed in the R- cells. Upregulation of cyclin D1 by EN is observed even in the absence of IGF-IR (see Figure 8). Upregulation of cyclin D1 is noted only in the cells expressing the EN fusion and does not change with serum stimulation.

With serum stimulation there is a marked increase in activation of Akt and Mek1/2 in the R+EN cells and R- cells however in the R-EN cells we do not note an

increase in Mek1/2 or Akt activation with serum stimulation. This data suggests that the IGF-IR axis may be important for activating the PI-3K-Akt and Ras-MAPK pathways in the presence of EN oncogene expression. In other words, cells expressing EN in the absence of IGF-IR appear to be somehow blocked from full activation of the Ras-MAPK and PI-3K-Akt pathways.

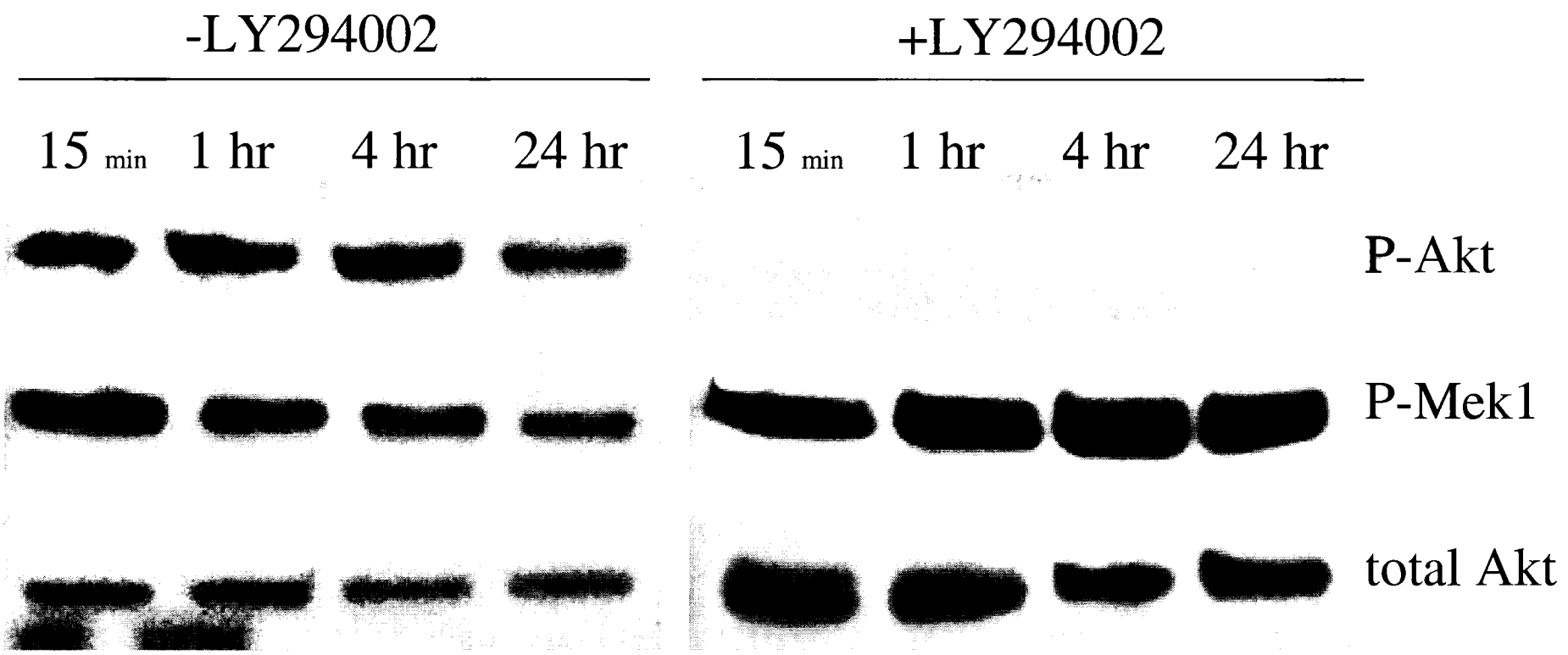
3.8 EN induces constitutive IRS-1 phosphorylation in NIH3T3 fibroblasts

The preceding experiments provide evidence that full activation of Mek1/2 and Akt by EN is dependent on the presence of IGF-IR. Basal activation of Akt and Mek1/2 by EN is, however, noted even in the absence of IGF-IR. The molecular adapter proteins that bind EN and lead to activation of Mek1/2 and Akt have not been identified. The consensus binding sites for Shc and p85 are not present within the NTRK3 portion of the EN fusion protein due to the position of the breakpoint. Previous work by our group has also demonstrated an inability of Grb2, Shc and p85 to bind EN (91)

The IGF-IR pathways that lead to activation of the Ras-MAPK and PI-3K-Akt pathways have been well characterized (57). The IGF-IR binds and tyrosine-phosphorylates a number of substrates including IRS-1-4, Gab-1, Shc, 14-3-3 (9), which lead to recruitment of other proteins that cause activation of proliferative and anti-apoptotic pathways mediated in part by the Ras-MAPK and PI-3K-Akt pathways (56).

Figure 7.

PI-3K inhibitors specifically block Akt activation. NIH3T3 expressing EN cells were grown in 9% CS/DMEM +/- 25μM LY294002 for the indicated time periods and then harvested for Western blotting. Blots were probed with antibodies specific for the phosphorylated forms of Mek1/2 and and Akt Ser 473. Equal protein loading was determined by probing the blot with an antibody against total Akt. LY294002 specifically inhibits activation of Akt and does not change levels of activated Mek1/2 or cyclin D1.


-LY294002
+LY294002
15 min
1 hr
4 hr
24 hr
15 min
1 hr
4 hr
24 hr
P-Akt
P-Mek1
total Akt

Given that the EN oncoprotein causes partial activation of the PI-3K-Akt and Ras-MAPK pathways in the absence of IGF-IR and that full, or high level, activation is found in cells engineered to re-express IGF-IR, we hypothesized that a component of the IGF-IR axis could perhaps be acting as a direct substrate for the EN fusion oncoprotein. In this way, such a molecule may link EN to activation of the downstream pathways. Some recent work has indicated that the insulin-receptor substrate-1 (IRS-1) may serve as this postulated link. Experimental work in other laboratories has demonstrated that IRS-1 acts as a direct substrate for TrkA and TrkB kinases (52, 95).

Yamada and co-workers recently reported (95) that NTRK2 stimulation is associated with increased IRS-1 phosphorylation and recruitment of the p85 regulatory subunit of PI-3K. As well, Miranda and co-workers have reported a direct association between TrkT1, a NTRK1 fusion oncoprotein, and IRS-1 (52). Based on the fact that EN activates the Ras-MAPK and the PI-3K-Akt pathways and that direct binding of p85 and Shc/Grb2 (to EN) has not been demonstrated (91), we hypothesized that IRS-1 could be acting as an adapter to activate these pathways by directly interacting with EN. Furthermore, an interaction between IRS-1 and EN could explain the partial transformation of the R- cells by EN as R-cells express a relatively high level of IRS-1 protein (9).

We first examined the tyrosine phophorylation status of IRS-1 in the presence and absence of EN in order to assess whether EN activates IRS-1 by tyrosine phosphorylation. We examined NIH3T3 cell lines expressing EN, and we noted a differentially tyrosine phosphorylated band at 180 kDa in the EN-expressing cells (Figure

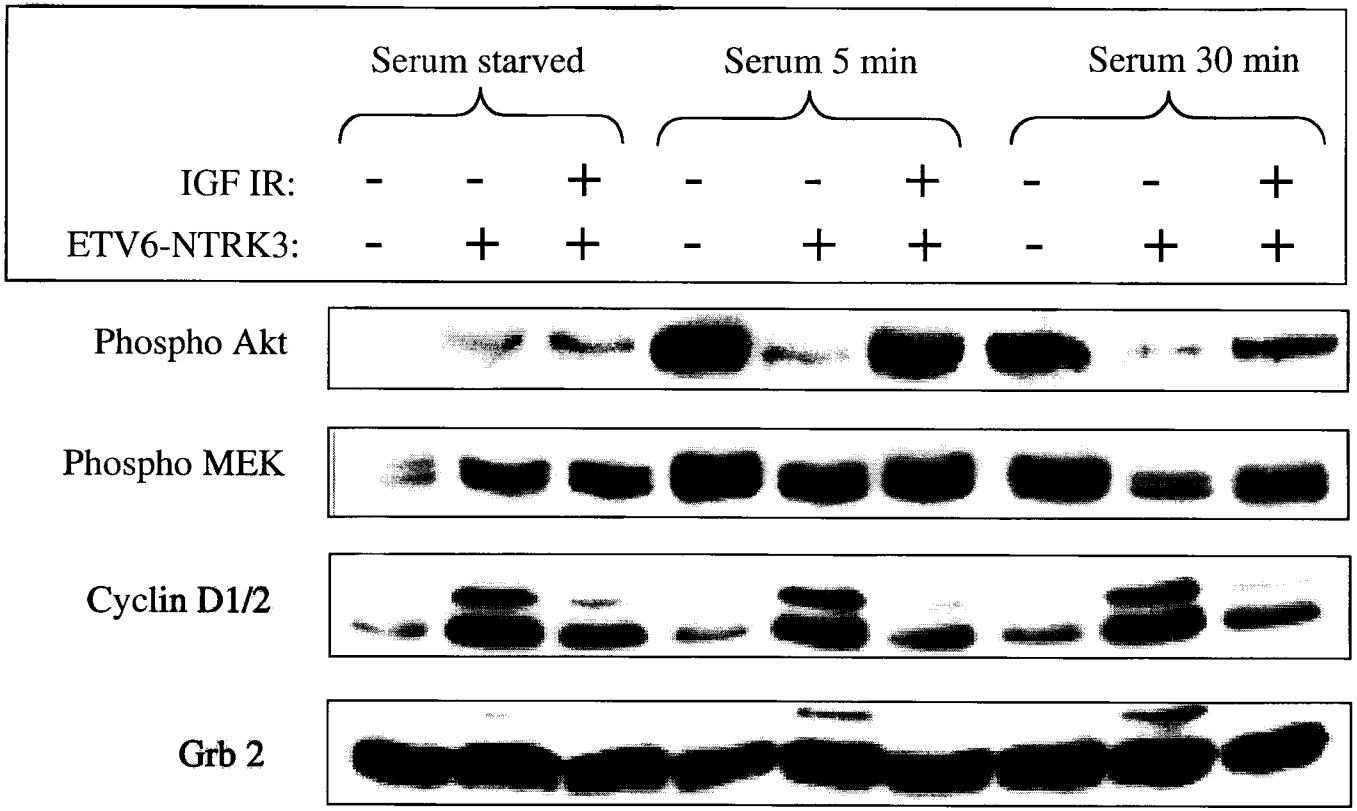
9 panel A). The upper panel of figure 9 shows that this same band becomes tyrosine phosphorylated in the vector control cells in response to serum and IGF-II stimulation. Re-probing of this blot with an IRS-1 specific antibody revealed that the differentially phosphorylated band at 180 kDa corresponds with IRS-1 (Figure 9, panel A). This indicates that IRS-1 is activated by tyrosine phosphorylation in a constitutive manner in EN-expressing cells. We confirmed the differential tyrosine phosphorylation of IRS-1 in EN expressing cells by immunoprecipitation. Figure 9 panel B illustrates the results from a typical experiment. IRS-1 is highly tyrosine phosphorylated in EN-expressing cells as compared to vector controls where tyrosine phosphorylation is not detectable under unstimulated conditions. We conclude that IRS-1 is differentially tyrosine-phosphorylated in 3T3 fibroblasts expressing EN. Our next objective was to determine whether the differential phosphorylation of IRS-1 linked with EN expression is dependent on the presence of IGF-IR.

3.9 EN expression causes constitutive IRS-1 tyrosine phosphorylation in the absence of IGF-IR

We examined the tyrosine phosphorylation of IRS-1 in R-EN cells in order to determine if constitutive tyrosine phosphorylation is dependent on the presence of IGF-IR. R-, R-EN, R+ and R+EN cell lines were cultured as previously described. Lysates were prepared for blotting with α-phosphotyrosine antibodies and the results are depicted in Figure 10 panel A. Once again, we note the presence of a tyrosine-phosphorylated band in the EN expressing cells at approximately 180 kDA. This band becomes tyrosine-

Figure 8.

Akt and Mek1/2 activation is attenuated in R-EN cells. In the absence of serum, R-EN and R+EN demonstrate constitutive low level activation of Akt (upper panel) and Mek1/2 (middle panel) as compared to R-cells. With serum stimulation Akt and Mek1/2 activation is greatly increased in R- and R+EN cell lines whereas there is no increase in the R-EN cell line. The levels of cyclin D1 are increased in the EN expressing cell lines. R-, R-EN and R+EN cell lines were cultured in 35mm dishes to 75% confluence and then placed in low serum (0.5%) for 18hrs. Cells were stimulated with 9% FBS and lysed at the indicated time points.


Serum starved
Serum 5 min
Serum 30 min
IGF IR: - - + - - + - - +
ETV6-NTRK3: - + + - + + - + +
Phospho Akt
Phospho MEK
Cyclin D1/2
Grb 2

phosphorylated upon stimulation by either serum or IGF-II in the R+ and R+EN cells. There is, however, no phosphorylation of this band noted in the R- cells, which is expected given that they lack IGF-IR. These blots were stripped and re-probed with α-IRS-1 antibody and to verify the identity of this band as IRS-1.

The constitutive phosphorylation of IRS-1 in the R- EN and R+EN cells as compared to R- control cells was confirmed by immunoprecipitation (Figure 10 panel B). This illustrates very low levels of tyrosine-phosphorylated IRS-1 in the control R- cells. In contrast, the R+EN and R-EN cells show approximately equally elevated levels of IRS-1 tyrosine phosphorylation. This indicates that IGF-IR is not necessary for IRS-1 tyrosine phosphorylation in EN-expressing cells.

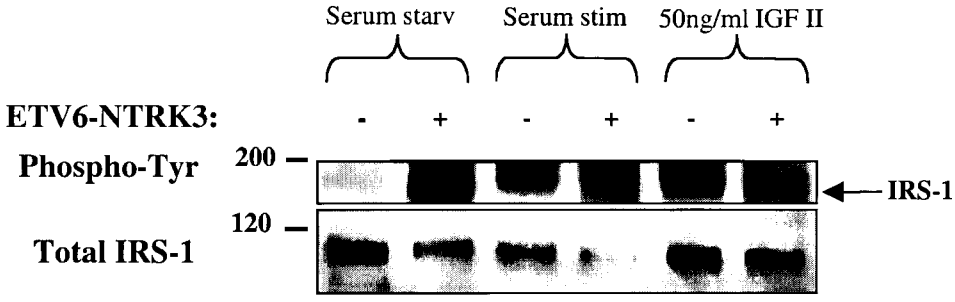
3.10 IRS-1 co-immunoprecipitates with EN

Based on the above findings and on the fact that EN has tyrosine kinase activity, we hypothesized a direct interaction between IRS-1 and EN. In order to test this hypothesis we carried out experiments to investigate whether IRS-1 could co-immunoprecipitate with EN. R+EN cells were lysed after stimulation with serum and lysates were used for immunoprecipitation with an α-HLH-Tel antibody. We detected the co-immunoprecipition of a tyrosine phosphorylated band at 180 kDa that after stripping and reprobing proved to be IRS-1 (Figure 11). We then demonstrated co-immunoprecipitation of EN with immunoprecipitaion of IRS-1, Figure 12 panel A. The characteristic tyrosine phosphorylated doublet of EN was pulled down with antibodies to IRS-1. When stripped and re-probed with an α-Trk antibody the identity of this doublet

Figure 9.

IRS-1 is constitutively tyrosine phosphorylated in EN-transfected NIH3T3 fibroblasts. (**A**) NIH3T3 +/- EN cells were cultured to 75% confluence, serum deprived for 18hrs (0.5% serum), and then stimulated with 9% serum or 50ng/ml of IGF-II. A differentially tyrosine phosphorylated band at approximately 180 kDA is noted in NIH3T3 EN lysates under serum deprived conditions compared to MSCV vector control cells. This band is phosphorylated in response to serum and IGF-II stimulation in the NIH3T3 vector alone control cells. This blot was stripped and the identity of this band was confirmed to be IRS-1. (**B**) Immunoprecipitation of IRS-1 from serum-starved NIH3T3 lysates +/- EN reveals increased basal levels of tyrosine phosphorylated IRS-1 in EN-expressing cells. Reprobing with an IRS-1 antibody reveals virtually equivalent levels of immunoprecipitated IRS-1.

A.


Serum starv
Serum stim
50ng/ml IGF II
ETV6-NTRK3:
- + - + - +
Phospho-Tyr
200
IRS-1
120
Total IRS-1

B. IP: IRS-1

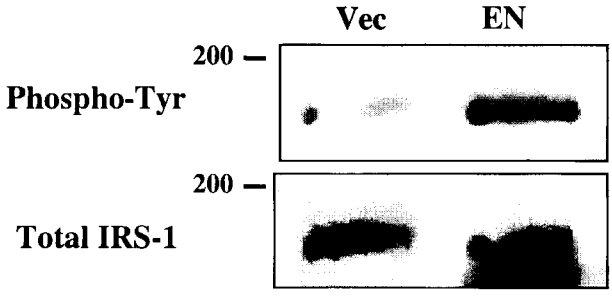
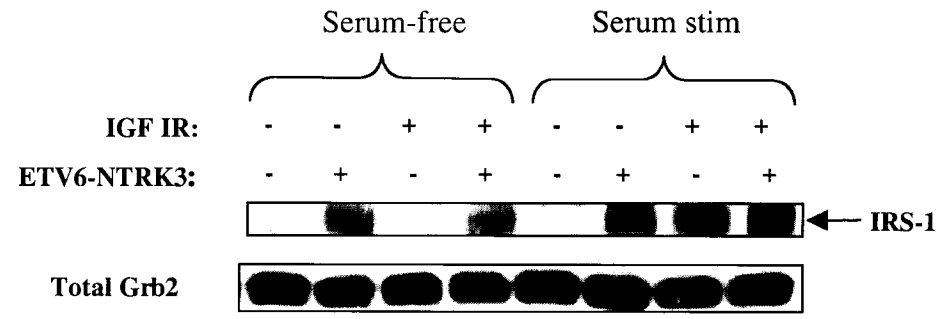
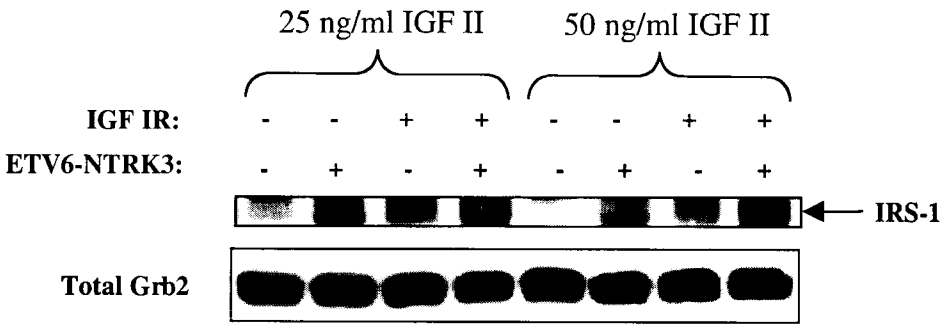

Vec
EN
200
Phospho-Tyr
200
Total IRS-1

Figure 10.

IRS-1 is constitutively phosphorylated in EN-expressing R- cells. (**A**) R-, R-EN, R+ and R+EN cells were cultured to 75% confluence and then serum deprived for 18hrs in 0.5% serum. Lysates were collected for phosphotyrosine Western blot analysis. A constitutively tyrosine phosphorylated band is noted at ~180kDa in the both the R+EN and R-EN cell lines. With IGF-II stimulation this band becomes tyrosine phosphorylated in the R+ cells but not in the R- MSCV control line. Stripping and reprobing with an IRS-1 specific antibody proved the identity of this band as IRS-1. Grb2 blotting was used to control for equal loading of protein samples. (**B**) Equal amounts of cell lysates (1000μg) from R-, R-EN and R+EN were collected for immunoprecipitation of IRS-1. Tyrosine phosphorylation of IRS-1 in R- cells was very low under both serum starvation and serum stimulation with 9% FBS for 30 minutes. Again, IRS-1 is found to be constitutively phosphorylated in EN expressing R+ and R-lines.

A. P-Tyr





B. IP:IRS-1

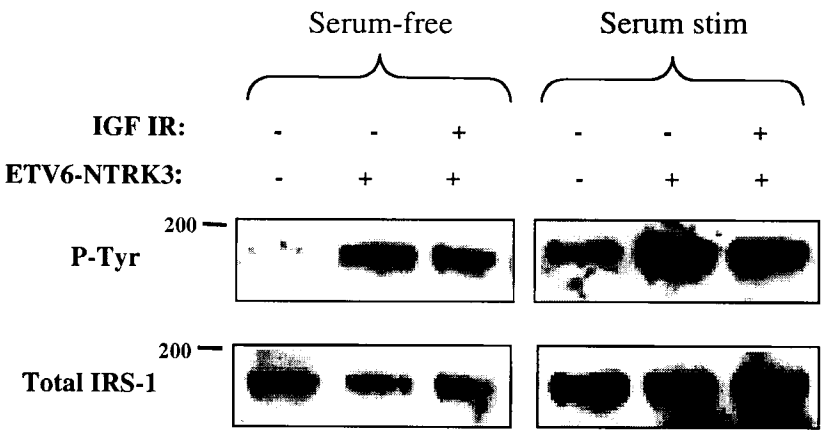
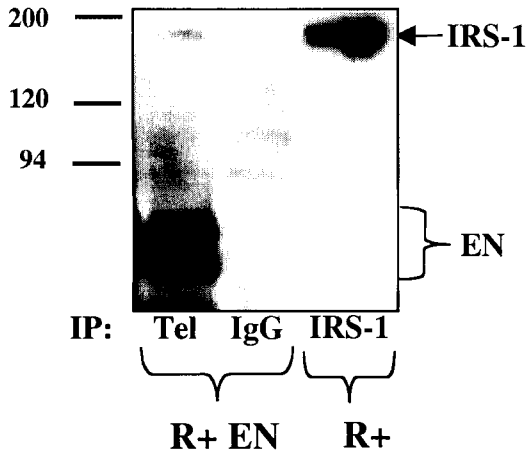


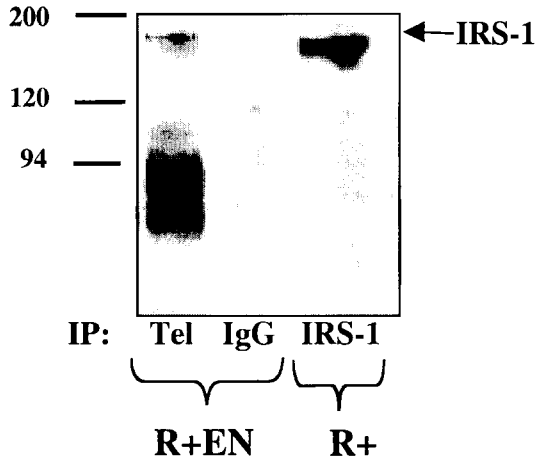
Figure 11.

EN co-immunoprecipitates with IRS-1. Immunoprecipitation of EN with α-HLH Tel antibody from R+EN lysates (1000μg) co-precipitates a tyrosine phosphorylated band of approximately 180 kDa (left panel). This band is identified as IRS-1 when the blot is re-probed with IRS-1 antibody. Immunoprecipitation of R+ lysate with an α-IRS-1 antibody serves as a positive control whereas incubation of R+EN lysates with normal rabbit IgG serves as a negative control.

P-Tyr Blot



IRS-1 Blot



was verified as EN (data not shown). These studies provide preliminary evidence that IRS-1 interacts either directly or indirectly with the EN protein.

3.11 Mutation of PLCγ-binding residue in EN reduces IRS-1 binding

To begin to attempt to map the IRS-1 binding site on EN we tested a set of tyrosine mutants involving the kinase domain of EN. We created the following tyrosine mutants; Y628Q (PLCγ-binding site), Y594F and Y560F.

We tested the ability of these three mutants to co-immunoprecipitate with IRS-1 The results in Figure 12, panel A demonstrate roughly equal amounts of EN are immunoprecipited with the α-HLH Tel antibody from each of the mutants. On the other hand, only a small amount of EN co-immunoprecipitates with IRS-1 from the Y628Q (PLCγ mutant)(see Figure 12, panel A, right side).

We then carried out a set of experiments to look at the absolute levels of tyrosine phosphorylated IRS-1 in the different EN mutants. We hypothesized that the PLCγ mutant would have lower levels of tyrosine phosphorylated IRS-1 as it appears to demonstrate a lower affinity for EN. Equal amounts of protein were used for immunoprecipition from each cell line. We found that levels of tyrosine-phosphorylated IRS-1 were roughly equal in the different mutants of EN with the exception of the K380N (kinase dead) which displays low levels of IRS-1 phosphorylation (see Figure 12, panel B).

3.12 EN causes increased tyrosine phosphorylation of IRS-1 and recruitment of Grb2 and p85

The results obtained from Western analysis of R-, R-EN and R+EN illustrate the importance of IGF-IR for high level activation of the PI-3K-Akt and Ras-MAPK pathways. In the absence of IGF-IR, there are lower levels of activation of Mek1/2 and Akt. Co-immunoprecipitation studies suggest a direct association between IRS-1 and EN. IRS-1 appears to be activated by tyrosine-phosphorylation by EN and therefore may represent a link between EN and activation of the PI-3K-Akt and Ras-MAPK pathways.

IRS-1 is an important adapter protein for the IGF and insulin signaling cascades. It becomes tyrosine-phosphorylated with IGF stimulation and gains the ability to recruit SH2-containing proteins such as PI-3K, Grb2/mSos and SHP-2 (32). These events in turn activate the downstream PI-3K-Akt and Ras-MAPK pathways. We therefore hypothesized that EN activation of IRS-1 leads to recruitment of Grb2 and p85 and that IGF-IR synergyzes with EN to increase activation of IRS-1 by increasing recruitment of p85 and Grb2. We hypothesized that this mechanism ultimately results in the high level activation of Akt and Mek1/2 that we observe in the R+EN and EN-expressing NIH3T3 cell lines.

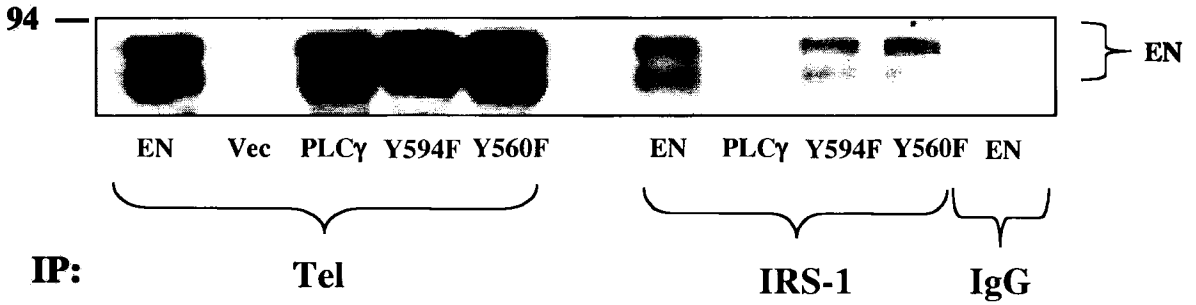
We carried out a set of experiments over the same time intervals as in Figure 6 in order to answer the following questions: Does the presence of IGF-IR cause increased or prolonged activation of IRS-1? Does the presence of IGF-IR cause increased recruitment of p85 and Grb2 ? We found, as shown before, that the levels of IRS-1 tyrosine-phosphorylation are increased in the presence of EN (Figure 13). Again, there does not

appear to be any difference in the levels of tyrosine phosphorylation of IRS-1 between the R-EN and R+EN cell lines in the absence of serum stimulation nor at any time point

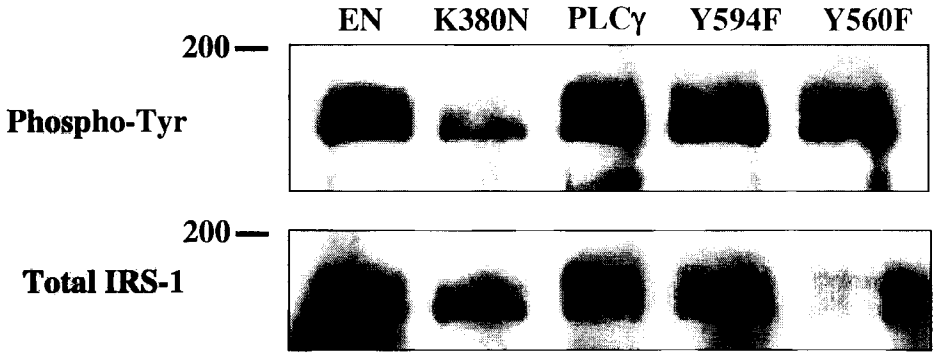
Figure 12.

Co-immunoprecipitation of EN with IRS-1 with IRS-1 is reduced with PLCγ-binding mutant. (A) Cell lysates (1000μg) were collected and subjected to immunoprecipitation with α-HLH Tel antibody as well as with α-IRS-1 antibody. Immunoprecipitates were washed three times in wash buffer and separated on 10% SDS PAGE and subjected to phospho-tyrosine blotting. A tyrosine phosphorylated doublet at 68 & 73 kDa is seen which corresponds with EN when blot is re-probed with α-TrkC antibody (data not shown). Tel immunoprecipites show the absence of EN from the control vector-transfected cells as well as co-immunoprecipitation of EN with IRS-1 IP. There is virtually no EN co-immunoprecipitating with the PLCγ (Y628Q) mutant whereas the levels of EN precipitating with the Y594F and Y560F mutants are equivalent. (**B**) NIH3T3 fibroblasts transfected with EN and various EN mutants: K380N kinase dead, Y594F mutant of tyrosine 594, Y560F mutant of tyrosine 560 and Y628Q mutant of the PLC γ-binding site. The K380N kinase dead mutant shows lower levels of IRS-1 phosphorylation as compared with wild type EN and the other mutants which show similar levels of activation. Cells were cultured in 9% calf serum/DMEM; 1000 μg of protein lysate was immunoprecipitated with 2.5ug of α-IRS-1 antibody. Immunoprecipitates were blotted with phospho-tyrosine antibody and subsequently α-IRS-1antibody to show total IRS-1.

A. P-Tyr Blot



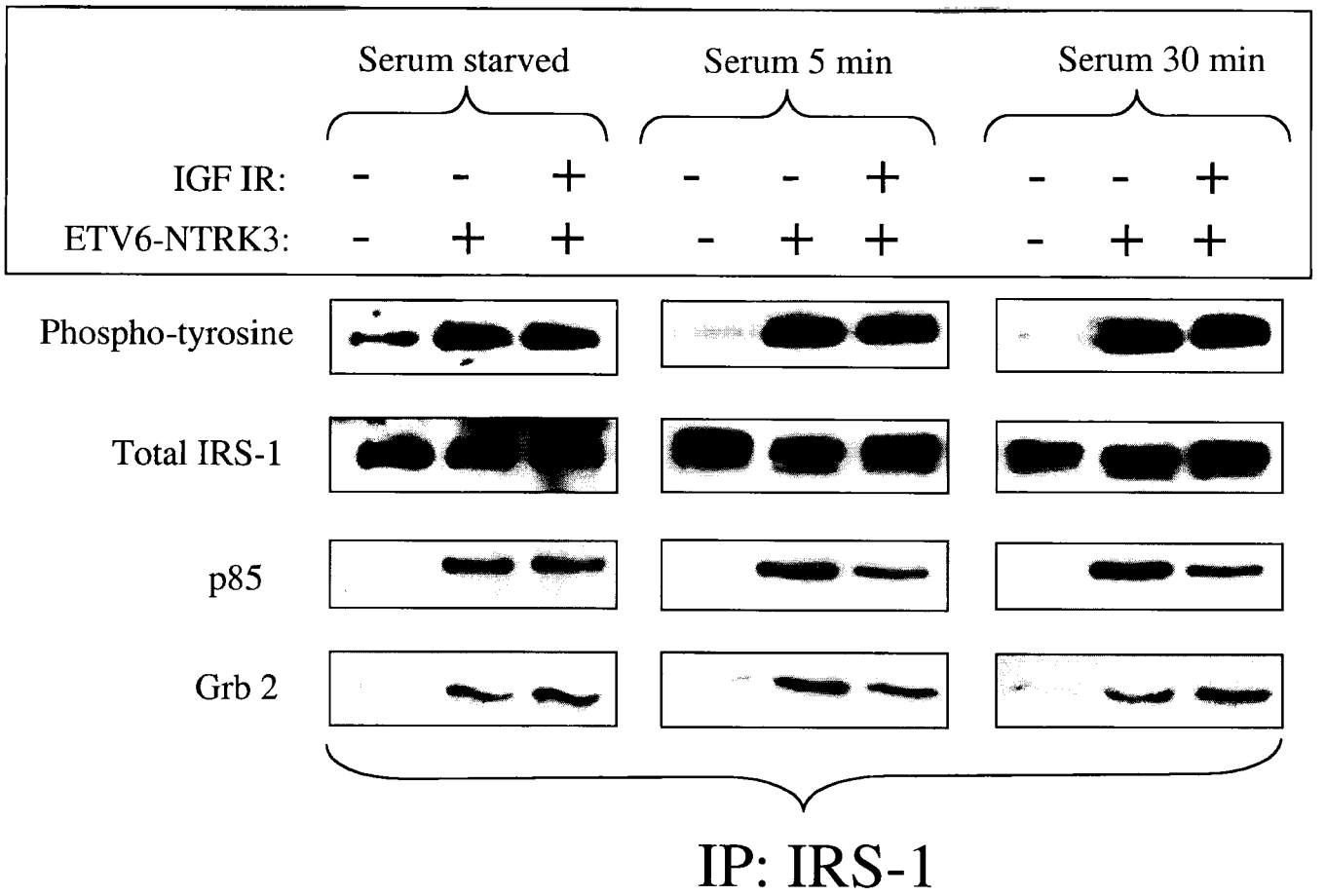
B. IP: IRS-1



following serum stimulation. Furthermore, we observe that the EN-induced increase in tyrosine phosphorylation of IRS-1 correlates with increased recruitment of p85 and Grb2 in both R- and R+ cells. The levels of Grb2 recruited by IRS-1 in the R-EN and R+EN are approximately equivalent. Therefore, EN activity results in activation of IRS-1 and recruitment of downstream components, p85 and Grb2, independently of IGF-IR. The level of IRS-1 activation and recruitment of downstream components does not correlate with either Mek1/2, Akt activation or with transformation. The mechanisms by which IGF-IR causes prolonged and high level activation of Mek1/2 and Akt remains unclear. This data however does suggest that IRS-1 may serve as a critical link to activation of the PI-3K-Akt and Ras-MAPK pathways.

Figure 13.

Tyrosine-phosphorylated IRS-1 recruits Grb2 and p85. R-, R-EN and R+EN cells were cultured to 75% confluency in 100mm dishes and then placed in 0.5% serum for 18 hrs. Subsequently they were stimulated with 9% FBS in DMEM and cell lysates were harvested at the indicated times. Equivalent amounts of protein (1000μg) were immunoprecipitated with α-IRS-1 antibody. The immunoprecipitates were then separated on 10% SDS PAGE. Phosho-tyrosine blotting demonstrates high levels of tyrosine phosphorylation of IRS-1 in the absence and presence of serum in the EN-expressing cells. The presence of IGF-IR is not required for phosphorylation of IRS-1 and there are similar levels of phosphorylation of IRS-1 in both R-and R+ cell lines The same blots were also probed for p85 regulatory subunit of PI3'K and Grb2. The highly phosphorylated forms of IRS-1 in the EN-expressing cell lines correspond to co-immunoprecipition of significant quantities of Grb2 and p85. Small amouts of Grb2 were found to co-immunoprecipitate with IRS-1 in the control cells however no p85 was detected in these co-immunoprecipitates.


Serum starved
Serum 5 min
Serum 30 min
IGF IR: - - + - - + - - +
ETV6-NTRK3: - + + - + + - + +
Phospho-tyrosine
Total IRS-1
p85
Grb 2
IP: IRS-1

CHAPTER 4

DISCUSSION

4.1 Overview and Conclusions

In this work we have illustrated two mechanisms by which growth factors play a role in tumorigenesis. We have demonstrated increased levels of IGF-II transcripts in CFS and cellular CMN. As well, we have investigated the mechanisms of action of ETV6-NTRK3, a transforming fusion oncogene found in CFS and cellular CMN. The ETV6-NTRK3 fusion oncoprotein has previously been found to dimerize and constitutively activate its tyrosine kinase function (91). Here, we provide evidence that EN induces transformation by activating IRS-1, a major downstream element of the IGF growth factor pathway. We provide evidence that EN induces constitutive activation of Akt and that transformation is dependent on activation of this pathway.

Our experiments indicate that the presence of IGF-IR is essential for EN-transformation despite the observation that IRS-1 is highly tyrosine-phosphorylated. A review of the possible mechanisms behind increased IGF-II expression as well as the role of IGF-IR in transformation will be presented in this discussion. Along with this, comments will be made on the novel findings that include the findings of constitutive activation of Akt and IRS-1 in EN-expressing cells.

4.2 IGF-II overexpression in CFS and CMN

Based on the near 100% correlation between the presence of ETV6-NTRK3 and the trisomy 11 we postulated that this extra copy of chromosome 11 carried some factor

important for tumorigenesis. The IGF-II gene is located along the p-arm of chromosome 11 and therefore we investigated IGF-II expression levels in CFS and CMN tumor samples.

IGF-II expression was of particular interest as it has been found to be implicated in a number of different pediatric malignancies including rhabdomyosarcoma, Wilm's tumor and neuroblastoma (86). The major source of circulating IGF-II is produced in the liver however it is also produced by most tissues and it is felt that it can act in an autocrine manner promoting cancer progression by increasing mitosis and resistance to apoptosis (94).

The finding of increased IGF-II expression lead to two primary questions:

1. What mechanisms are involved in increased IGF-II expression ?
2. What is the role of increased IGF-II expression in EN-mediated transformation ?

4.3 Mechanisms of increased IGF-II expression

The possible mechanisms leading to increased IGF-II expression include: biallelic expression resulting from loss of imprinting of the maternal allele, increased gene copy number, or increased gene transcription secondary to the influence of upstream enhancer elements.

4.3.1 IGF-II gene imprinting

The IGF-II gene is located in the 11p15.5 region which is imprinted. Imprinting is a phenomenon that involves differential methylation of genomic DNA in the germ line that causes silencing of either a maternal or paternal gene (55). The IGF-II gene is

maternally silenced and under normal circumstances only expressed from the paternal allele (94). In certain malignancies there is a loss of the imprinting status and as a result, both copies of the gene are expressed (84). This results in increased expression of the affected gene.

In seven cases of CFS and CMN that were examined in our lab there is no evidence for loss of imprinting of the IGF-II gene (M.Garnett, Vancouver, unpublished data,). In other words, the maternal copy of the IGF-II gene appears to be transcriptionally silent in the cases of CFS and CMN examined.

4.3.2 VNTR polymorphism

A specific variable-number tandem repeat (VNTR) DNA polymorphism has been recently identified upstream of the IGF-II and insulin genes in the 11p15.5 region. Other VNTR polymorphisms are associated with human disorders such as malignancy. In certain conditions VNTR polymorphisms can function to influence gene expression. In the case of the *Ha-ras* oncogene there is a downstream VNTR which acts to influence expression (44).

The 5' insulin VNTR polymorphism is grouped into three types based in its size. The different alleles range in size: class I allele from 0.4-0.9 kb, class II 1.2 kb, and class III> 3kb. It is hypothesized that the VNTR may function as part of a nuclear matrix attachment region that affects chromatin structure and accessibility of transcriptional factors (5). In this model it is felt that the shorter VNTR alleles mediate less attachment to the nuclear matrix and as a result allow greater exposure of the IGF-II gene to transcriptional factors. Work by C. Deal has demonstrated increased levels of IGF-II

mRNA are associated *in vivo* and *in vitro* with the type I VNTR allele in placental tissue and hepatocyte cultures (61, 87). In some work done in collaboration with C.Deal we looked at the genotypes of CMN and CFS tumors that overexpressed IGF-II.

The results from this work demonstrate a statistically significant larger proportion of tumors that are type I homozygous (94%) as compared to control samples (54%) (M-H Iglesias, Montreal, unpublished data). These results are very intriguing in that they suggest the existence of an inheritable mechanism that predisposes to increased IGF-II production and associated malignancy. Further work is necessary however, to attempt to further correlate the genotype data with tumor IGF-II levels and clinical outcome. Important work is also being undertaken to characterize the mechanisms by which the VNTR alters downstream expression of the IGF-II.

4.4 EN transformation requires Akt activation

In this work we have presented novel findings that support a critical role for Akt activation in EN-induced transformation. The serine/threonine kinase, Akt, has found to be important for transformation and tumorigenesis based on data from *in vitro* studies and from the study of primary tumor samples. Overexpression of Akt has been found in ovarian cancer (17), pancreatic cancer (18) and breast cancer (10). Akt is a component of the phosphatidyloinositol 3' kinase (PI-3K) survival pathway, in which both PI-3K and it's negative regulator, PTEN, are upstream of Akt and are directly regulated by growth factor tyrosine kinases.

The role of PI-3K as an upstream mediator of survival signaling initially became evident through the use of specific blocking agents such as LY294002 which block PI-3K

activation and induce apoptosis (90). Involvement of the PI-3K-Akt pathway in tumorigenesis has been underlined by the discovery of numerous mutations of upstream and downstream pathway components in human tumors. As well, mutations of PTEN, a negative regulator of PI-3K, have been found in a number of cancers including breast, prostate, kidney and glioblastoma (47).

Akt is a 55-60 kDa protein that contains a pleckstrin homology (PH) domain that binds to 3'phosphorylated lipids and localizes Akt to the membrane where it is activated by phosphorylation of residues serine 473 and threonine 308 (1). The presence of the PH domain and recruitment to the cell membrane is essential for activation of Akt (81).

Given the importance of this survival signaling pathway in transformation we investigated the effect of EN expression on activation of Akt. The data presented illustrate constitutive activation of Akt by EN and that blocking this activation reverts transformation. Furthermore, the attenuated activation of Akt in the absence of IGF-IR and in the presence of constitutively phosphorylated IRS-1 is an intriguing finding that suggests perhaps that IGF-IR may serve to attract activated IRS-1 and EN to the cell membrane such that they comes into close proximity with the upstream elements of this pathway.

4.5 IGF-IR is required for EN transformation

The results of this work illustrate the importance of the IGF signaling pathway for EN-mediated transformation. Despite being able to activate IRS-1, the major signaling substrate of IGF-IR, EN induces only partial transformation of IGF-IR-knockout fibroblasts. In the following sections a discussion of the importance of the IGF-IR

knockout cell line as a model system for investigating IGF signaling in transformation is presented. Following this, we will discuss the significance of the data that suggests a direct interaction between IRS-1 and EN. Finally, we will speculate about the function of the IGF-IR, and why it is required for transformation in this setting.

4.6 Transformation of IGF-IR knockout cells

Because the IGF axis has been closely implicated in the development of many different forms of pediatric and adult malignancy there has been significant focus on understanding the intracellular signaling cascades that emanate from IGF-IR. Using the IGF-IR knockout murine fibroblast model (R- cells) system, significant work has been done to understand the importance of IGF IR in contributing to transformation by specific oncogenes. Using this system, it has been possible to isolate the contributions of the IGF axis in activating specific transforming signaling pathways. Several oncogenes such as *Ha-ras*, SV 40 large tumor antigen, EWS-FLI1 and overexpressed growth factors such as PDGF, EGF have been unable to transform R-cells (21, 78, 85). Two oncogenes that have been reported to transform R-cells are *v-src* and a GTPase-deficient mutant of $G_{\alpha 13}$, a heterotrimeric G-protein family member (49).

Work done by Valentinis (89) studying the transforming properties of *v-src* and *c-src* has lead to the characterization of some of the important structural and signaling aspects of the IGF-IR in transformation. Firstly, this work illustrates that an activated tyrosine-phosphorylated IRS-1 along with an activated oncogene, *c-src*, may not be sufficient alone to induce transformation in the absence of IGF-IR. Both *v-src* and *c-src* cause equivalent constitutive levels of tyrosine-phosphorylation of IRS-1 whereas only *v-*

src is able to cause transformation of the R- cells as measured by colony formation in soft agar. Subsequent work has confirmed that overexpression of IRS-1 alone does not induce transformation of R- cells (7).

The importance of the C-terminal portion of IGF-IR in transformation was also discovered in this work. By creating mutations of IGF-IR and re-expressing them in *c-src*-expressing R-cells, it was found that a small region at the C-terminus of IGF-IR is required for the receptor to be competent for transformation. These findings suggest the existence of a specific signaling pathway that originates from the C-terminus of IGF-IR that is essential for transformation. The ability of *v-src* to transform R- cells may relate to an ability to activate this pathway or through its ability to activate the STAT or FAK pathways (89).

It has been found that the presence of a C-terminal region is essential for transformation not only by *c-src* but by many oncogenes (57). In particular, the serine residue 1283 of IGF-IR has been found to mediate a critical anti-apoptotic signal required for transformation (57). This residue is known to bind 14-3-3 proteins and mediate mitochondrial translocation of Raf1, a serine-threonine kinase downstream of Ras in the Ras-MAPK pathway, which leads to inactivation of the pro-apoptotic protein, Bad.

In recent work by Baserga, this IRS-1-independent anti-apoptotic pathway was characterized in 32D cells. In work done with different IGF-IR mutants it was found that transfected IGF receptors that lacked the 1280-1283 serine residues fail to translocate Raf1 to the mitochondria and as a result cause release of cytochrome C and apoptosis (66). This work re-enforces the concept that activation of IRS-1 alone does not lead to transformation in R-cells and that it may be the presence of this C-terminal portion of the

receptor that is required to stimulate an anti-apoptotic pathway that is required for transformation (66).

4.7 Insulin Receptor Substrate-1 (IRS-1) interacts with ETV6-NTRK3

The characterization of IRS-1 as an interactor with the EN oncoprotein is a novel and interesting finding. Prior to this work the mechanisms by which EN activates the downstream Ras-MAPK pathway were unknown. In the preceding experiments we have provided evidence that IRS-1 is highly tyrosine-phosphorylated in the presence of EN and that this phosphotyrosine-dependent activation induces recruitment of upstream elements of the pathways that are essential for EN transformation.

Recent work by other groups has revealed evidence that indicate that IRS-1 is a direct substrate for other NTRK family members, namely NTRK1 (95) and NTRK2. In work done by Yamada and co-workers, NTRK2 activation was found to result in increased IRS-1 tyrosine phosphorylation and recruitment of downstream elements of the PI-3K-Akt pathway. Work focussed on studying TrkT1, a chimeric fusion oncoprotein isolated from thyroid cancer, has revealed that both IRS-1 and IRS-2 interact with the NTRK1 portion of this chimeric fusion (52) and cause activation of downstream signaling cascades. To date, there have been no reports of NTRK3 interacting with IRS-1. The significant amount of homology between the NTRK family members makes the existence of an EN-IRS-1 association very plausible.

In this work we have provided evidence that the mutation of the PLCγ-binding site reduces EN association with IRS-1. This result is consistent with some of the work that was done with the TrkT1 fusion oncoprotein (52). Using a yeast-two hybrid system,

Miranda and co-workers were able to isolate the specific tyrosine residues on the NTRK1 portion of the fusion that are involved in binding with IRS-1. This yeast-two hybrid analysis reveals a 50% reduction in the interaction between IRS-1 and TrkT1 with mutation of tyrosine 499, which corresponds to the PLCγ-binding residue in EN.

The domain structures of the IRS proteins (consisting of IRS-1-4) also reveal specific domains that could be implicated in NTRK3 binding. All IRS's contain an N-terminal pleckstrin homology (PH) domain, which binds membrane phospholipids or acidic motifs, followed by a phosphotyrosine-binding (PTB) domain which interacts with phosphotyrosine residues in the sequence: asparagine-proline-glutamic acid-phosphotyrosine (NPEY). The C-terminal portion of the IRS proteins contain numerous potential tyrosine phosphorylation sites. These sites are less conserved amongst the IRS family of proteins. Although there is no NPEY site in EN, due to the site of the translocation breakpoint, the PLCγ site has a very similar architecture. The architecture of this site is: TYGK. It remains to be fully confirmed if the PLCγ-binding site is in fact within the IRS-1 binding site.

4.8 IGF-IR mediates complete transformation

4.8.1 IRS-1 subcellular location

There is accumulating evidence that the IRS proteins exist in dynamic equilibrium between the cytoplasm and inner cell membrane subcellular compartments. The N-terminal pleckstrin homology (PH) domain of IRS-1 has been found to be responsible for translocating IRS-1 from the cytoplasm to the inner cell membrane during insulin and IGF stimulation (69). As well, the presence of the PH domain has been shown to be essential for efficient tyrosine phosphorylation of IRS-1 with IGF stimulation (54, 98).

There is also evidence to suggest that the subcellular location of IRS-1 is important not only for its ability to become tyrosine phosphorylated but also for its ability to activate downstream pathways. In a set of experiments using a myristylated IRS-1 it was found that constitutive membrane localization of IRS-1 correlates with decreased p85 binding but at the same time with increased Akt and Ras-MAPK activation (43).

In the work we have presented in this thesis we observe decreased levels of Akt and Mek activation in the IGF-IR knockout cell lines. Activation of these pathways appears to be refractory to serum stimulation. In the co-immunoprecipitation experiments we also note slightly decreased p85 interacting with IRS-1 from R+ cell line. Based on this data and the data from the reviewed work we speculate that the presence of IGF-IR may play a role in localizing IRS-1 to the inner cell membrane where it can efficiently activate components of the PI-3K-Akt and Ras-MAPK pathways.

4.8.2 IGF IR –specific pathways

IGF-IR is required for EN-mediated transformation despite the fact that EN itself induces high levels of activated IRS-1 in the absence of IGF-IR. We have speculated that IGF-IR may play a role in translocating activated IRS-1 to the inner cell membrane to an environment that is enriched for upstream elements of the PI-3K-Akt and Ras-MAPK pathways. It is also possible that IGF-IR stimulates pathways that are independent of IRS-1. There is some evidence to support this concept.

The insulin-receptor substrate-1 binds IGF-IR at tyrosine 950 and becomes tyrosine-phosphorylated. As already described, IRS-1 then recruits downstream proteins that activate PI-3K and Ras. Mutational analysis of IGF-IR has identified specific regions

of IGF IR that are required for mitosis, prevention of apoptosis and for transformation (57). C-terminal-truncation mutants have been found to promote mitosis but not allow transformation (7). Differential display analysis studying the C-terminal mutant has identified a potential tumor-suppressor gene, DICE-1 (deleted in lung cancer), that is suppressed by the C-terminus (93). The suppression of DICE-1 by IGF IR may be essential for EN-mediated transformation.

Site-directed mutagenesis studies have revealed that the serine residues 1280-83 of IGF-IR are also essential for transformation, as discussed above (section 4.7) (48). This pathway is another IRS-1-independent pathway that has been found to be essential for transformation. Again, in the absence of IGF-IR these pathways may not be stimulated resulting in the inability of EN to induce transformation.

Another possible reason for the requirement of IGF-IR for transformation is that it enhances activation of IRS-1-stimulated pathways. It is possible that without the synergistic activation of the PI-3K-Akt and Ras-MAPK pathways by both IRS-1 and IGF-IR ,through common adapters, that a certain threshold level of activation is not achieved and transformation does not occur. It has been well documented that IGF-IR itself can activate the Ras-MAPK pathway by direct binding of Shc to tyrosine 950 (9). As well, direct activation of the PI-3K pathway is mediated by p85 binding directly at tyrosine 1316 of IGF IR (57). Yet another speculated mechanism that may contribute relates to the possibility that there may exist specific tyrosine residues on IRS-1 that are uniquely phosphorylated by IGF-IR and not by EN and that these residues recruit and activate distinct and unique downstream signaling cascades that are required to mediate EN-induced transformation.

CHAPTER 5

SUMMARY AND CONCLUSIONS

We have demonstrated that trisomy 11 correlates with increased expression of IGF-II transcripts in EN-expressing cases of CFS and CMN. In an *in vitro* system we provide evidence that the IGF pathway is required for transformation and for high level activation of the EN-associated transformation pathways: PI-3K-Akt and Ras-MAPK. This work illustrates that EN transformation is not only dependent on upregulation of the Ras-MAPK pathway but that it is also dependent on constitutive activation of the PI-3K-Akt pathway. We present evidence that IRS-1 acts as a major adapter molecule for EN and functions to recruit downstream proteins Grb2 and p85 involved in activating the PI-3K-Akt and Ras-MAPK pathways.

Future goals of this research will focus on understanding the role of IGF-IR in mediating transformation as well as on further characterizing the molecular domains involved in the putative IRS-1–EN interaction. The specific residues and domains of EN involved in binding IRS-1 will be investigated using IRS-1 and EN mutants in the mammalian-two-hybrid assay in conjunction with in-vitro GST pull-down experiments. More specifically, the possible role of IGF-IR in localizing EN and IRS-1 to the inner cell membrane will be addressed using immunofluoresence and confocal microscopy. As well, alternative pathways downstream from IGF-IR will be investigated with respect to their levels of activation in IGF-IR knockout and 3T3 cell lines expressing EN.

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