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Tyrosine Protein Kinase Expression in Long-Term Quiescent WI-38 Cells Following Growth Factor Simulation

Aleksandra Korcz[1], Dianne Robert Soprano[2], and Kenneth J. Soprano[3]

[1,3]Department of Microbiology and Immunology (A.K., K.J.S.), [2]Department of Biochemistry (D.R.S.), and [2]Fels Institute of Cancer Research and Molecular Biology (D.R.S., K.J.S.), [3]Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Abstract We have used the WI-38 cell long-term quiescent model system to study the regulation of cell cycle progression at the molecular level. By modulating the length of time that WI-38 cells are density arrested, it is possible to proportionately alter the length of the prereplicative or G-1 phase which the cell traverses after growth factor stimulation in preparation for entry into DNA synthesis. Stimulation of long- and short-term density arrested WI-38 cells with different growth factors or higher concentrations of individual growth factors does not alter the time required by long-term cells to enter S after stimulation. However, the time during the prereplicative period for which these growth factors are needed is different. Long-term quiescent WI-38 cells require EGF to traverse the G-0/G-1 border but do not need and apparently cannot respond to IGF-1 during the first 10 h after EGF stimulation, the length of the prolongation of the prereplicative phase. This suggests that EGF stimulation of long-term quiescent WI-38 cells initiates a series of molecular events which make these cells "competent" to respond to the "progression" growth factor, IGF-1. In light of the well-established role of protein tyrosine kinases in signal transduction, we set out to identify, clone, and analyze the expression of receptor and non-receptor tyrosine kinases which potentially could play a role during the prolongation of the prereplicative phase in making the long-term quiescent WI-38 cells competent to respond to IGF-1. We obtained 49 clones representing 11 different receptor and non-receptor type protein tyrosine kinases. Analysis of expression of these clones revealed a variety of different patterns of expression. However, the most striking pattern was exhibited by IGF-1 receptor. Our results suggest that induction of IGF-1 receptor mRNA by EGF may be an important event in the establishment of competence by EGF in long-term density arrested WI-38 cells.

Key words: WI-38 cells, protein tyrosine kinases, IGF-1 receptor, growth factors, EGF

We have used the WI-38 cell long-term quiescent model system to study the regulation of cell cycle progression at the molecular level [for review, see Soprano, 1994]. By modulating the length of time that WI-38 cells are density arrested, it is possible to proportionately alter the length of the prereplicative or G-1 phase which the cell traverses after growth factor stimulation in preparation for entry into DNA synthesis [Augenlicht and Baserga, 1974; Owen et al., 1987, 1989, 1990].

As a first step in using this model system to identify and understand growth regulatory events, we previously determined the qualitative and quantitative growth factor requirements necessary to stimulate G-1 progression and entry into S in long-term quiescent WI-38 cells [Owen et al., 1989]. We found that stimulation of long- and short-term density arrested WI-38 cells does not involve different individual growth factors or different concentrations of the same growth factors. The same defined medium supplemented with EGF, IGF-1, and dexamethasone at the same concentrations stimulated DNA synthesis with an efficiency and kinetics similar to that elicited by fetal calf serum in both short- and long-term quiescent WI-38 cells. Moreover, use of different growth factors or higher concentrations of individual growth factors did not alter the time required by long-term cells to enter S after stimulation. However, the time during the prereplicative period for which these

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Address reprint requests to Dr. Kenneth J. Soprano, Department of Microbiology & Immunology, Temple University School of Medicine, 3400 North Broad Street, Philadelphia, PA 19140.

growth factors are needed was different. Two groups of experiments lead us to this conclusion: those in which serum was withdrawn at various times after stimulation and those in which individual growth factors were withheld for various lengths of time at the time of stimulation. Short-term quiescent WI-38 cells can, do, and in fact must respond either immediately or very early during the G-0/G-1 transition to both EGF and IGF-1, since a delay in the addition of either growth factor results in a delay in the initiation of DNA synthesis (i.e., a prolongation of the prereplicative phase). Likewise, our finding that no additional prolongation occurred in long-term quiescent WI-38 cells when addition of IGF-1 was delayed up to 10 h or when addition of EGF was delayed up to 3 h suggested that long-term quiescent WI-38 cells were unable to respond to EGF during the first 3 h after stimulation and could not respond to IGF-1 during the first 10 h after stimulation (the length of the prolongation of the prereplicative phase).

Most recently, we have shown that the reduction in EGF responsiveness that occurs during long-term growth arrest results from a change in the nature and/or location of the EGF receptor such that they bind substantially less ligand and internalize what little they bind at a slower rate [Donigan et al., 1993]. Interestingly, we have found that long-term quiescent WI-38 cells require approximately 3 h to achieve internalized levels of EGF comparable to those measured within 2 min of treatment of short-term quiescent WI-38 cells. This is consistent with our previous finding that long-term quiescent WI-38 cells are unable to respond to EGF during the first 3 h after stimulation. It is possible that a critical level of EGF must be bound and internalized in order to initiate "competence," the signal transduction pathway which induces the molecular events which subsequently allow the cell to respond to "progression" growth factors such as IGF-1.

Tyrosine protein kinases (TPKs) are characterized as either receptor-type or non-receptor-type enzymes. Those of the receptor-type bind polypeptide hormones and growth factors via their extracellular sequences and initiate transmission of intracellular signals by activation of their cytoplasmic catalytic domains [for reviews see Moxham and Jacobs, 1992; Claesson-Welsh, 1994]. The non-receptor type represent a diverse collection of kinases that are grouped together because of their lack of extracellular domains. However, these TPKs associate with cellular membrane proteins and, like their receptor-type counterparts, facilitate cell surface initiated signal transduction [for reviews see Bolen, 1993; Fantl et al., 1993]. In light of the role of TPKs in mediating growth factor signal transduction, we set out to identify, clone, and analyze the expression of receptor and non-receptor tyrosine kinases which potentially could play a role during the prolongation of the prereplicative phase in making the long-term quiescent WI-38 cells competent to respond to IGF-1.

MATERIALS AND METHODS

Preparation of Short- and Long-Term Quiescent WI-38 Cells

Stock cultures of WI-38 human diploid fibroblast were obtained from a generous gift of Dr. Vincent Cristofalo (Medical College of Pennsylvania) at passage 12. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere with 10% CO_2. Age of the WI-38 cells used in all experiments was estimated to be between passages 16 and 18 (Population Doubling Level 40–45) as determined by the method of thymidine incorporation [Cristofalo and Sharf, 1973].

As described previously [Owen et al., 1990; Soprano, 1994], density-dependent growth arrest in WI-38 cells can be divided into two stages: the first, which occurs during the first 7–10 days after plating, is called "early or short-term" quiescence and exhibits no prolongation of the prereplicative stage. The second stage, called "prolonged or long-term" quiescence, occurs 11 days or more following growth arrest (or 18 days after plating). The prereplicative stage following stimulation of these cells was progressively prolonged.

WI-38 cells were routinely plated at a density of 2×10^4 cells/cm^2 in 100 mm tissue culture dishes (Corning Glass Works, Corning, NY) in DMEM supplemented with 10% FBS. This plating medium was replaced with DMEM supplemented with 0.1% FBS on days 10, 16, and 23 after plating in order to prevent experimental artifacts created by either amino acid deficiency or toxic pH. It should be noted that we and others [Augenlicht and Baserga, 1974; Miska and Bosmann, 1980] have shown that re-feeding with DMEM supplemented with 0.1% FBS does not alter the time or kinetics of G-1 progression

into S following long-term, density-dependent growth arrest. For all of the experiments described here, short-term quiescent cells were assayed at 12 days after plating while long-term quiescent cells were analyzed at 26 days after plating. In each case, quiescent cells were stimulated with DMEM supplemented with either EGF (50 ng/ml) or EGF (50 ng/ml) followed 10 h later by IGF-1 (50 ng/ml) [Owen et al., 1989].

Entry into DNA synthesis as determined by autogradiography was routinely used as an assay for growth factor responsiveness. As we have previously reported [Owen et al., 1987, *1990*], short-term quiescent WI-38 cells entered DNA synthesis within 18–24 h after stimulation with either 10% fetal calf serum or purified growth factors (EGF + IGF-1). The Labeling Index for the experiments reported here ranged between 50–60%. Long-term quiescent WI-38 cells stimulated with either fetal calf serum or purified growth factors routinely entered DNA synthesis between 28 and 34 h and typically exhibited a Labeling Index of 45–50%. Treatment of long-term quiescent WI-38 cells with EGF alone failed to stimulate DNA synthesis (Labeling Index = $<2\%$).

Isolation of Cellular RNA

WI-38 cells were harvested by trypsinization and total RNA isolated using the RNAzol reagent (TM Cinna Scientific Inc., Friendswood, TX). RNA was quantitated by absorbance at 260 nm and purity was assessed by absorbance at 280 nm. Integrity of each RNA sample was confirmed by analysis on agarose-formaldehyde gels stained with ethidium bromide as described previously [Cosenza et al., 1988; Owen et al., 1987, 1990; Toscani et al., 1987]. For the experiments reported here, we routinely used 2 μg of total RNA for each RNAse protection assay (see below).

RT-PCR and Cloning

Before cDNA synthesis, RNA samples were treated with Message Clean Tm Kit (Gene Hunter Corp., Brookline, MA). The aliquots (0.6 to 2 μg) of the DNA-free RNA were used for cDNA synthesis with Superscript reverse transcriptase (Gibco, Grand Island, NY) and random hexamer primers (Pharmacia, Piscataway, NY) according to Sambrook et al. [1989]. Fifty percent of cDNA reaction was used as a template in PCR reaction using mixtures of oligonucleotide primers, degenerate at the underlined positions, derived from the consensus sequences of the conserved kinase subdomains of known TPKs [Wilks, 1989, 1991]. The sequence of these primers, which also contained BamHI and HindIII restriction sites was the following:

PTK I: $^{5'}$CGCGGATCCACA(C)GNGAC(T)C-(T)T $^{3'}$ and
PTK II: 5'AGAGAAGCTTCCAA(T)AG(T)GAC-CAG (C)ACG(A)T(C)C$^{3'}$.

Thirty cycles of PCR were carried out using Taq DNA polymerase (Promega, Madison, WI) using an automated DNA thermal cycler (Coy Lab Products, Ann Arbor, MI). Each cycle consisted of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C. The aliquots of PCR products were analyzed on a 10% polyacrylamide gel to confirm that the length of the fragments was approximately 200–230 bp. Next, the total mixtures of PCR products from different reactions were cleaved with BamHI and HindIII and electrophoresed in 10% polyacrylamide gel. Ethidium bromide stained bands (200–230 bp) were cut out, eluted, and cloned into pGEM 3zf (Promega). Forty-nine recombinant clones were obtained. Nucleotide sequences were determined by chain-termination sequencing method. DNA sequences were analyzed using GCG package version 6.0. The sequences were searched against non-redundant nucleotide sequence database(s) using the BLAST programs [Altschul et al., 1990] implemented in e-mail server.

RNAse Protection

Steady state levels of mRNA were analysed by RNAse protection as described previously [Cosenza et al., 1988; Owen et al., 1987, 1990; Toscani et al., 1987] with the following minor modification. Immediately after synthesis of in vitro transcripts from PTK clones the probes were dissolved in hybridization buffer containing formamide and run on 15 cm high 5% polyacrylamide gel at 400 V until the bromophenol blue dye reached the bottom of the gel. After exposure of the gel on film, the pieces of gel representing a single band for every probe were cut out. Elution of anti-sense RNA from crushed slices of gel was performed overnight in 4°C in buffer containing: 0.5 M NH_4 acetate, 0.1 mM Na_4 EDTA, 1 mM $MgCl_2$, 0.1% SDS, followed by phenol/chloroform extraction of supernatant and final precipitation with 3 volumes of ethanol. The quality of purified probes was tested again on a 10% polyacrylamide mini gel.

Since the expression of β_2-microglobulin has been shown to remain constant in cells subjected to a variety of stimuli including addition of growth factors and antisense oligodeoxynucleotides, it was used as a standard to normalize for variation in RNA concentration from sample to sample [Cosenza et al., 1988; Owen et al., 1987, 1990; Toscani et al., 1987; Carter et al., 1991; Peña et al., 1993]. Appropriate exposures of the gels were densitometrically scanned and quantitated using the Hoeffer GS-365 System. Data obtained after normalization to β_2-microglobulin levels were plotted as optical density relative units vs. time after stimulation.

RESULTS AND DISCUSSION

Our approach to studying the role of tyrosine kinases in making EGF stimulated long-term quiescent WI-38 cells competent to respond to IGF-1 involved the application of the RT-PCR technique using a mixture of degenerate primers derived from the consensus sequences of the conserved kinase domains of known TPKs [Wilks, 1989, 1991]. Total RNA was isolated from long-term quiescent WI-38 cells at various times following treatment with EGF and in cells treated for 10 h with EGF followed by IGF-1 for 1 h. These RNAs were then used in reverse transcriptase polymerase chain reactions (RT-PCR). First, cDNA was prepared using random hexamer primers. Next the cDNA was amplified by PCR using primers containing conservative sequences specific for the highly conserved regions flanking the catalytic domain of protein tyrosine kinases. These PCR fragments were then cloned into pGEM 3zF. We obtained 49 clones. The inserts of each clone were sequenced and the region between the amplimers corresponding to the catalytic domain was compared at both the nucleotide and amino acid level to known protein tyrosine kinase sequences found in the Gene Bank. We considered a clone identical to a previously known protein tyrosine kinase if its sequence had more than 90% identity over a stretch of approximately 180 base pairs or 60 amino acids. The 49 clones represent 11 different, previously identified protein tyrosine kinases. Alignment of the amino acid sequences spanning the catalytic domain of each of the 11 different clones can be found in Table I. While a number of both receptor type and non-receptor type protein tyrosine kinases were identified, we were somewhat surprised that we did *not find* any unique, previously unknown protein tyrosine kinases and that the number of different clones we obtained was limited to only 11. We can conclude only that it is indicative of the TPK composition of the WI-38 cell line or of the physiological status of the cells when they were assayed. We did not analyze actively proliferating WI-38 cells. It certainly is possible that another, more diverse group of TPKs may be present in cells which have not been growth arrested.

Tyrosine Protein Kinases (TPKs) Identified in WI-38 Cells by RT-PCR

Table II shows that the receptor type TPKs found in WI-38 cells consisted of: IGF-1 receptor, PDGF receptor, *flg* (fibroblast growth factor receptor), and three recently cloned TPKs which establish new subfamilies of receptor type tyrosine kinases: *axl*, *tek*, and *tkt*. The IGF-1, PDGF, and FGF receptors are cell surface receptors which are extremely well characterized and their role in regulating cell proliferation has been studied extensively [for reviews see Baserga 1985; 1993; Claesson-Welsh, 1994; Fantl et al., 1993].

TABLE I. Sequence Alignment of Tyrosine Protein Kinases Cloned From WI-38 Cells

	10	20	30	40	50	58
Jak1	XARNVLVESE	HQVKIGDFGL	TKAIETDKEY	YTVKDDRDSP	VFWYAPECLX	QSKFYIAS
FD22	XARNVLVESE	HQVKIGDFGL	TKAIETDKXY	YTVKDDRDSP	VFWYAPECLI	QCKFYIAS
tyk2	AAXNVLLDND	RLVKIGDFGL	AKAVPEGHEY	YRVREDGDSP	VFWYAPECLK	EYKFYYAS
lynB	RAANVLVSES	LMCKIADFAL	ARVIEDN.EY	TAREG.AKFP	IKWTAPEAIN	FGCFTIKS
c-abl	XARNCLVGEN	HLVKVANFXL	NRLMTED.TY	TAHAG.AKFP	IKWTAPESLA	YNKFSIKS
IGF1-R	AARNCMVAED	FTVKIGDFGM	TRDIYET.DY	YRKGGKGLLP	VRWMSPESLK	DGVFTTYS
tkt	ATRNCLVGKN	YTIKIADFGM	SRNLYSG.DY	YRIKGRAVLP	IRWMSWESIL	LGKFTTAS
tek	AARNILVGEN	YVAKIANFGL	SR...GQ.EV	YVKKTMGRLP	VRWMAIESLN	YSVYTTNS
flg	AARNVLVTED	NVMKIADFGL	ARDIHHI.DY	YKKTTNXRLP	VKWMAPEALF	DRIYTH..
axl	AXRNCMLNEN	MSVCVADFGL	SKKIYNG.DY	YRQGRIAKMP	VKWIAIESLA	DRVYTSKS
PDGFR-1	AARNVLICEG	KLVKXCDFXL	ARDIXXD.SN	YISKXSTFXP	LKWMXPESIF	NNVYTTLT

TABLE II. Receptor Type TPKs Identified in WI-38 Cells

Name	No. clones isolated	% Identity	Description	Gene bank accession no.
IGF-1-R	7	92–100	Human insulin-like growth factor receptor precursor	P08069
PDGF-R	8	90–100	Human platelet-derived growth factor (PDGF) receptor mRNA	M21616
tek	2	98–100	*H. sapiens* receptor protein tyrosine kinase *tek* mRNA	L06139
axl	1	98–100	Human tyrosine kinase receptor *axl*, complete	M76125
tkt	1	98	*H. sapiens* mRNA for receptor protein tyrosine kinase	X74764
flg	1	98	Human mRNA for fibroblast growth factor receptor Ig-domain	X57119-X57122

The more recently discovered members of the receptor type TPKs are not as well studied. *Axl* was originally isolated from DNA of patients with chronic myelogenous leukemia [O'Bryan et al., 1991] but its expression can be detected in the majority of cell types examined which suggests that *axl* might be important in normal cellular activity. The *axl* gene encodes a 140 kDa protein which is capable of transforming NIH 3T3 cells. On the basis of amino acid sequence it is most similar to the insulin receptor family. However it is structurally unique in that *axl* contains two immunoglobulin-like domains and two fibronectin type III domains in its extracellular region [Fantl et al., 1993].

Tek was cloned by RT-PCR from murine embryonic heart [Dumont et al., 1992] and was found to be most closely related to FGF-R and to the product of the *c-ret* protooncogene. *Tek* defines a new subfamily of receptor TPKs characterized by three different types of structural motifs: immunoglobulin(Ig)-like loops; cysteine-rich EGF-like repeats, and fibronectin type III repeats. Like *axl*, it encodes a 140 kDa protein. *Tek* protein and/or transcripts are detectable in highly vascularized embryonic tissues and also in some cell lines of endothelial origin [Dumont et al., 1993].

Tkt is a novel receptor type TPK most closely related to the neurotrophin receptor, *trk*. It exhibits 47–49% identity to *trk* within the kinase domain [Karn et al., 1993]. The characteristic feature of *tkt* is the presence of a factor VIII domain in its extracellular region and the absence of any other domains typical of receptor type tyrosine kinases. *Tkt* mRNA is expressed at high levels in heart and lung. Lower levels of *tkt* were detected in human brain, placenta, liver, muscle, and kidney [Karn et al., 1993].

As can be seen in Table III, non-receptor type TPKs identified in EGF-stimulated WI-38 cells were represented by: *lyn B*, *c-abl*, Jak1, *tyk2*, and FD22 (mouse tyrosine kinase). *Lyn B* which was originally cloned from a human placenta cDNA library belongs to the *src* family of non-receptor TPKs, showing closest similarity to *lck* tyrosine kinase within this group [Yamanashi et al., 1987]. Although *lynB* mRNA was found to be expressed at various levels in placenta and in different human fetal tissues (brain, lung, liver, kidney) it was not detected previously in cultured human embryo fibroblast cells [Yamanashi et al., 1987].

The product of the *c-abl* protooncogene is a ubiquitously expressed TPK previously found in both resting and actively growing cells [Welch and Wang, 1993]. It is localized primarily to the nucleus but may be found in the cytoplasm when overexpressed [Sawyers et al., 1994]. Because the *c-abl* tyrosine kinase has an actin binding domain, binds DNA and can also form a complex with retinoblastoma protein in cell cycle-dependent manner [Welch and Wang, 1993], it is hypothesized that *c-abl* may be involved in some type of signal transduction that couples the status of cytoskeleton with growth regulatory gene transcriptional regulation [Bolen, 1993].

Jak1 and *tyk2* are members of a new class of non-receptor TPKs characterized by the presence of a second phosphotransferase-related domain immediately N-terminal to the TPK domain and lack of SH domains [Wilks et al., 1991; Firmbach-Kraft, 1990]. They were originally

TABLE III. Non-Receptor Type TPKs Identified in WI-38 Cells

Name	No. clones isolated	% Identity	Description	Gene bank accession no.
c-abl	2	91	Human *c-abl*	M14752 K00009 P00519
lyn B	1	90–96	Human *lyn B* mRNA encoding a tyrosine kinase	M16038 M79321
tyk2	2	98–100	Human *tyk2* mRNA for non-receptor TPK	X54637 P29597
Jak1	16	98–100	Jak1 human TPK	P23458
FD22	4	98–100	Mouse protein tyrosine kinase	M33425

cloned and isolated from hematopoietic cells but were found to be widely expressed in both hematopoietic and non-hematopoietic cell lines as membrane associated phosphoproteins of approximately 130 kDa. Jak1 and *tyk2* represent interferon responsive cellular kinases. Jak1 is required for both interferon -alfa/beta and -gama signal transduction pathways whereas *tyk2* is essential for the interferon alfa response [Muller et al., 1993].

Fd22 is a mouse tyrosine kinase originally isolated from a murine hematopoietic cell line by the RT-PCR strategy [Wilks, 1989]. It has not yet been characterized.

Expression of Receptor Type Tyrosine Protein Kinases Following EGF Treatment

Once we obtained this representative group of protein tyrosine kinases we next wished to determine how they were expressed in growth factor stimulated short-term and long-term quiescent WI-38 cells. In order to distinguish each of the protein tyrosine kinases from one another, we employed the RNAse protection method. This technique allows us to distinguish between closely related TPKs since even a single base pair of mismatched sequence will result in cleavage of the protected band. ^{32}P-labeled riboprobes were prepared from each clone and used to determine the pattern of RNA expression of the various protein tyrosine kinases in either short-term quiescent WI-38 cells stimulated with EGF or long-term quiescent WI-38 cells stimulated with EGF alone or EGF followed after 10 h by IGF-1.

We first examined the expression of receptor-type protein tyrosine kinases. Figure 1A and B show the pattern of expression obtained for PDGF-R and *tek* following EGF treatment of long-term quiescent WI-38 cells. Steady state levels of PDGF-R and *tek* remained constant during the 10 h following EGF stimulation and then gradually increased (to ~1.5 to 2.0-fold) upon further treatment with EGF. This increase was not observed when EGF treatment was followed at 10 h with IGF-1. Figure 1C shows that the pattern observed using *trk* as a probe was slightly different. While levels of *trk* mRNA were more variable, they did not change significantly for up to ~15 h following EGF treatment. However, *trk* mRNA levels did increase ~2-fold following addition of IGF-1 at 10 h. Expression of each of these receptor-type protein tyrosine kinases was found to be constant in short-term quiescent WI-38 cells stimulated with EGF (data not shown). Moreover, the levels observed were approximately equal to those detected in long-term quiescent WI-38 cells.

Figure 1D and E is a graphic representation of the results obtained using probes specific for *flg* and *axl*. In contrast to our previously described results, for each of these growth factor receptor-type tyrosine kinases, we observed a slow, gradual increase in steady state mRNA levels during the first 10 h of EGF treatment of long-term quiescent WI-38 cells. By 10 h, the time when the cells have presumably reached "competence," levels of these receptor protein tyrosine kinases were ~2-fold higher than that detected in the unstimulated cells. Levels of *flg* continued to rise and by 15 h after EGF treatment, reached a level ~3-fold greater than the level detected in quiescent cells. Treatment of the competent WI-38 cells at 10 h with IGF-1 resulted in a rapid, significant decrease of *flg* to levels approximately 25% lower than observed in the quiescent cells. The decrease in *axl* mRNA levels was

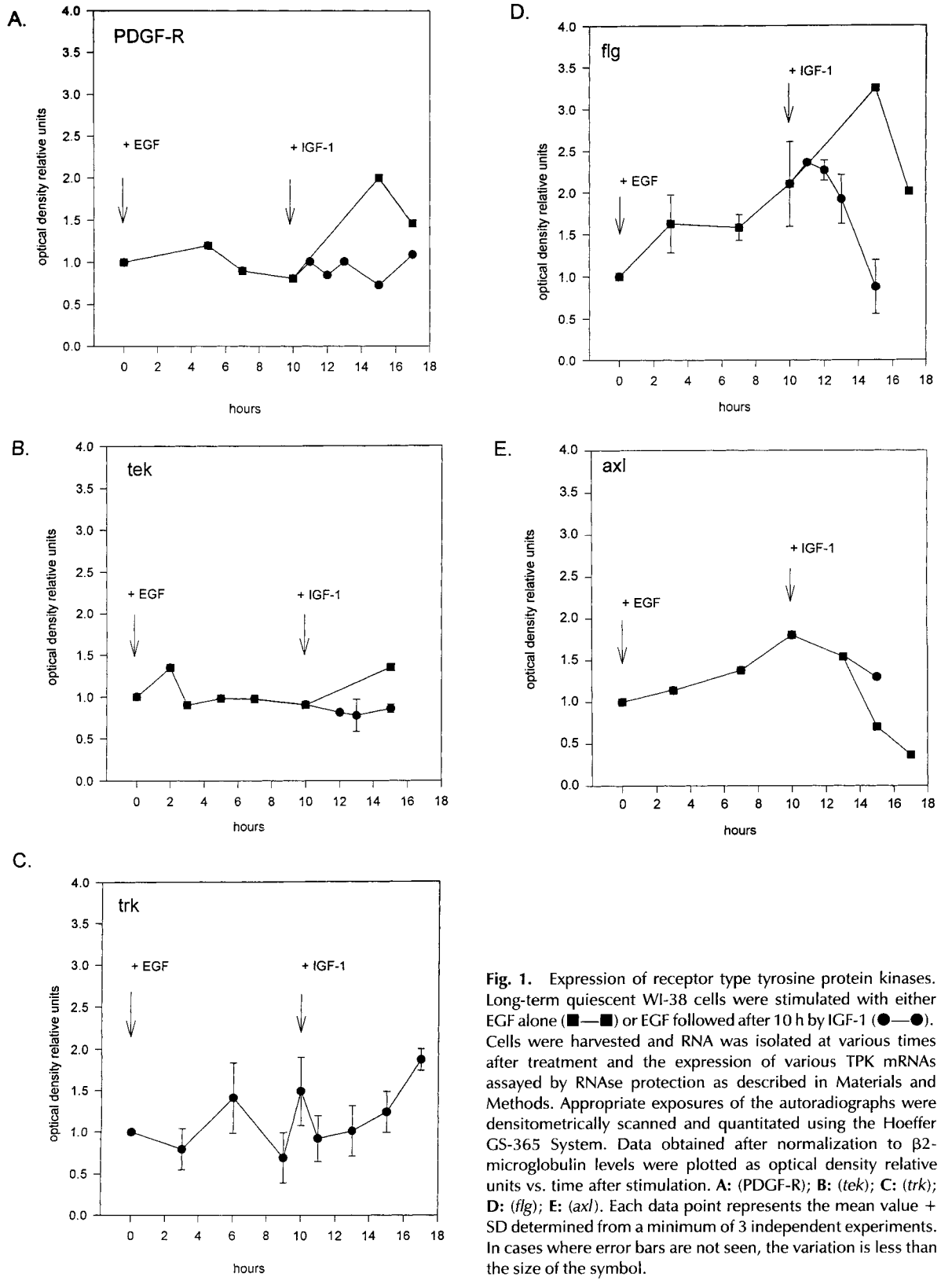


Fig. 1. Expression of receptor type tyrosine protein kinases. Long-term quiescent WI-38 cells were stimulated with either EGF alone (■—■) or EGF followed after 10 h by IGF-1 (●—●). Cells were harvested and RNA was isolated at various times after treatment and the expression of various TPK mRNAs assayed by RNAse protection as described in Materials and Methods. Appropriate exposures of the autoradiographs were densitometrically scanned and quantitated using the Hoeffer GS-365 System. Data obtained after normalization to β2-microglobulin levels were plotted as optical density relative units vs. time after stimulation. **A:** (PDGF-R); **B:** (*tek*); **C:** (*trk*); **D:** (*flg*); **E:** (*axl*). Each data point represents the mean value + SD determined from a minimum of 3 independent experiments. In cases where error bars are not seen, the variation is less than the size of the symbol.

more gradual and more modest, reaching levels approximately equal to that detected in the quiescent cells.

We also examined the expression of these protein tyrosine kinases in short-term quiescent WI-38 cells. Expression of *flg* and *axl* was relatively constant in the short-term quiescent WI-38 cells stimulated with EGF and the levels detected were only slightly lower than those found in long-term quiescent WI-38 cells (data not shown).

Figure 2A shows that in long-term quiescent WI-38 cells stimulated with EGF, IGF-1 receptor mRNA amounts gradually increased to a peak level which was ~2.3-fold higher than the G-0 level. This peak level was achieved by 10 h, the time at which the cells were "competent." As was true of many of the other receptor TPKs, IGF-1-R mRNA levels decreased as the cells proceeded into G-1. This was true whether or not IGF-1 was added at 10 h.

However, a different, more interesting pattern was observed when the expression of IGF-1-R was analyzed in short-term quiescent cells and compared to the pattern exhibited in their long-term quiescent counterparts. Figure 2B shows that the short-term quiescent WI-38 cells (which are already "competent" to respond to IGF-1) expressed levels of IGF-1R mRNA 2–3-fold higher than the long-term quiescent WI-38 cells. This higher level was comparable to that detected after the long-term quiescent WI-38 cells had been treated with EGF for 10 h, the time at which they become competent (see Fig. 2A). As was true of expression observed in the long-term quiescent cells, levels of IGF-1-R decreased rapidly upon treatment with IGF-1, as the cells progress through G-1.

Thus a variety of patterns were observed for expression of the various receptor-type tyrosine kinases. However, comparison of these patterns as well as the levels between short- and long-term quiescent WI-38 cells suggests that for the most part, there is no correlation between expression of receptor-type protein tyrosine kinases and establishment of competence by EGF. These results suggest that while a basal level of these receptor protein tyrosine kinase mRNAs can be detected in WI-38 cells, expression is not regulated at least at the steady state RNA level by length of growth arrest or manner of stimulation. The only possible exception is the IGF-1-R which was expressed in a manner consistent with the possibility that increases in the levels of this transcript might be at least one event in the establishment of competence by EGF (see Summary and Conclusions).

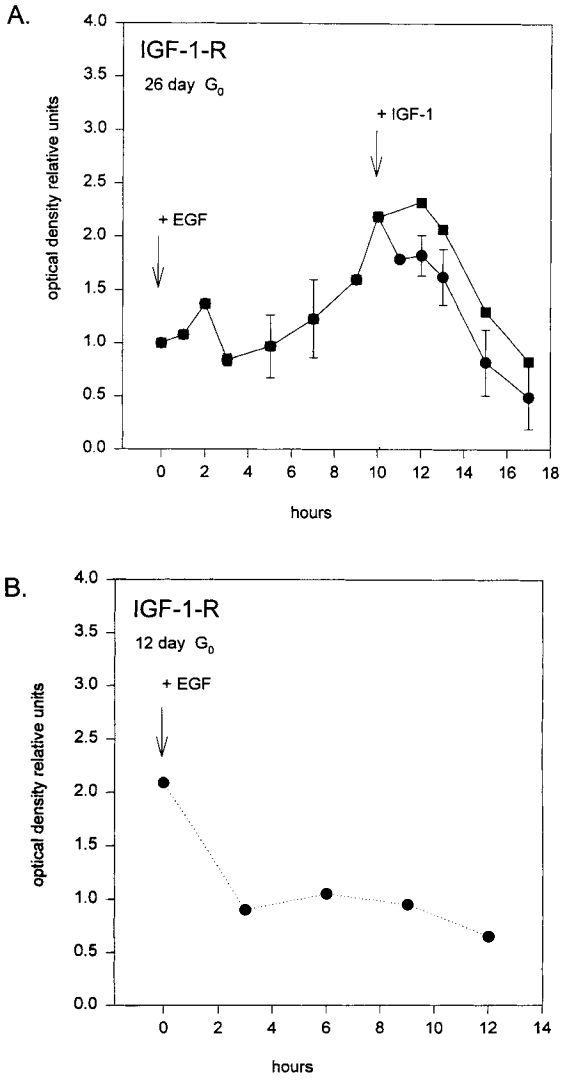
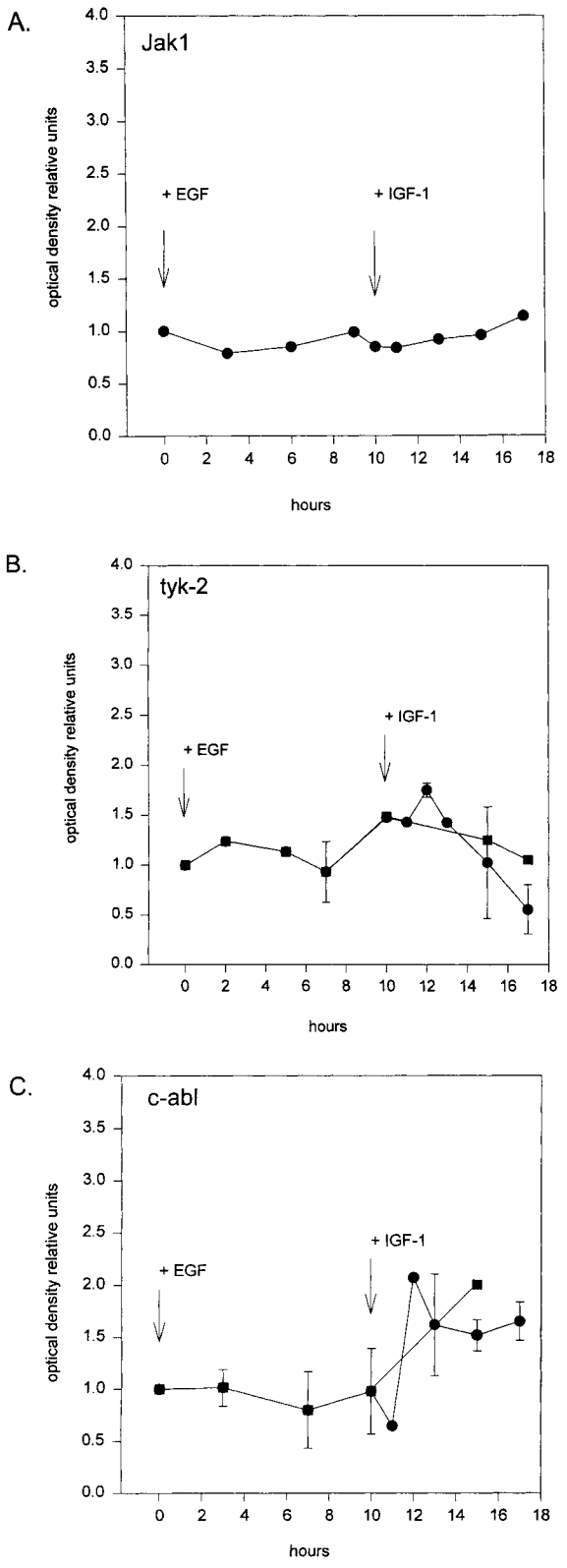


Fig. 2. Expression of IGF-1 receptor mRNA following EGF treatment of long-term quiescent WI-38 cells. Long-term (26-day) quiescent (**A**) or short term (12-day) quiescent (**B**) WI-38 cells were stimulated with either EGF alone (■—■ or (●—●) or EGF followed after 10 h by IGF-1 (●—●). At various times after treatment, cells were harvested, RNA isolated and analyzed by RNAse protection as described for Figure 1 and in Materials and Methods. Values presented for EGF stimulated short-term (12-day) cells were normalized to the amount detected in unstimulated long-term (26-day) quiescent cells.

Expression of Non-Receptor Type Tyrosine Protein Kinases Following EGF Treatment

Figure 3A–C shows the results of RNAse protection analysis of the expression of Jak1, *tyk2*, and *c-abl*. It can be seen that Jak1 expression



was not altered by addition of either EGF or IGF-1 (Fig. 3A). In the case of *tyk2,* Figure 3B shows that that levels of this non-receptor TPK increased slightly (~ 1.5-fold) by 10 h after EGF treatment, then dropped to below the G-0 level after stimulation with EGF for 10 h followed by IGF-1 for 2 h. Figure 3C shows that *c-abl* mRNA levels remained constant until 10 h following EGF stimulation. After that time, we detected a 1.5–2.0-fold increase in *c-abl* steady state mRNA levels. Unlike many of the other TPKs examined, *c-abl* levels do not rapidly drop with prolonged growth factor treatment but rather remain elevated.

Expression of all three non-receptor TPKs were found to be relatively constant in the EGF stimulated short-term quiescent cells. Levels were comparable to those found in long-term quiescent WI-38 cells prior to stimulation (data not shown).

It should be noted that neither *lynB* nor FD22 were detected by RNAse protection in either short-term or long-term quiescent WI-38 cells. Since we had originally cloned each of these PTKs from these cells by RT-PCR, it is likely that these transcripts are expressed in WI-38 cells but are present in amounts below the limits of detection by RNAse protection.

Summary and Conclusions

Analysis of expression of 11 TPK clones following growth factor stimulation of short- versus long-term quiescent WI-38 cells revealed a variety of different patterns of expression. However, the most striking pattern was exhibited by IGF-1 receptor. Our results suggest that induction of IGF-1 receptor mRNA by EGF may be an important event in the establishment of competence by EGF in long-term density arrested WI-38 cells. It should be noted that a number of other studies have suggested that cell cycle progression is regulated by the IGF-1-R. We and others have shown that if either 3T3 or WI-38 cells are primed with PDGF or EGF, a delay in the addition of IGF-1 results in a proportional delay in the entry of cells into S phase [Cristofalo et al.,

Fig. 3. Expression of non-receptor type tyrosine protein kinases. Long-term quiescent WI-38 cells were stimulated with either EGF alone (■—■) or EGF followed after 10 h by IGF-1 (●—●). At various times after treatment, cells were harvested, RNA isolated and analyzed as described in the legend to Figure 1 and in Materials and Methods. **A:** (Jak1); **B:** (*tyk2*); **C:** (*c-abl*).

1989; Owen et al., 1990; Yoshinouchi and Baserga, 1993]. Likewise, it has been known for a number of years that treatment of quiescent cells with growth factors such as PDGF and EGF will result in a 2–3-fold increase in the number of IGF-1 binding sites [Clemmons and van Wyk, 1981; Clemmons and Shaw, 1983]. Also, Reiss et al. [1992] have shown that IL-2 induces a large increase in levels of IGF-1-R mRNA in T lymphocytes. Most recently, Baserga and colleagues [Pietrzkowski et al., 1992; Baserga, 1993] have shown that 3T3 cells which constitutively overexpress both IGF-1 and the IGF-1-R can grow in serum-free medium, whereas if the endogenous IGF-1-R is deleted, no growth is observed even when the growth medium is supplemented with either fetal calf serum or individual growth factors normally sufficient to maintain growth [Sell et al., 1994]. Our data, presented in this report, combined with these previous studies provide strong evidence that the number of IGF-1 receptors is critical for regulation of cell cycle progression through G-1 and that growth factors (such as PDGF or EGF) render cells "competent" to progress through G-1 by increasing the number of IGF-1 receptors, thereby rendering them capable of responding to IGF-1.

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REFERENCES

Altschul SF, Gish W, Miller W, Myers EW, Lipmann DJ (1990): Basic local alignment search tool. J Mol Biol 215:403–410.

Augenlicht LH, Baserga R (1974): Changes in the G-0 state of WI-38 fibroblasts at different times after confluence. Exp Cell Res 89:255–262.

Baserga R (1985): "The Biology of Cell Reproduction." Cambridge: Harvard University Press.

Baserga R (1993): The double life of the IGF-1 receptor. Receptor 2:261–266.

Bolen JB (1993): Nonreceptor tyrosine protein kinases. Oncogene 8:2025–2031.

Carter R, Cosenza SC, Pena A, Lipson K, Soprano DR, Soprano KJ (1991): A potential role for c-jun in cell cycle progression through late G-1 and S. Oncogene 6:229–235.

Claesson-Welsh, L. (1994): Platelet-derived growth factor receptor signals. J Biol Chem 269:32023–32026.

Clemmons DR, Shaw DS (1983): Variables controlling somatomedin production by cultured human fibroblasts. J Cell Physiol 115:137–142.

Clemmons DR, van Wyk JJ (1981): Somatomedin: Physiological control and effect on cell proliferation. In Baserga R (ed): "Tissue Growth Factors." Berlin: Springer Verlag, pp 161–208.

Cosenza SC, Owen TA, Soprano DR, Soprano KJ (1988): Evidence that the time of entry into S is determined by events occurring in early G-1. J Biol Chem 263:12751–12758.

Cristofalo VJ, Sharf BB (1973) Cellular senescence and DNA synthesis. Exp Cell Res 76:419–427.

Cristofalo VJ, Philips PD, Sorger T, Gerhard G (1989): Alterations in the responsiveness of senescent cells to growth factors. J Gerontol 44:55–62.

Donigan AM, Cavalli RC, Pena A, Savage CR, Soprano DR, Soprano KJ (1993): Epidermal growth factor receptors lose ligand binding ability as WI-38 cells progress from short-term to long-term quiescence. J Cell Physiol 155: 164–170.

Dumont DJ, Yamaguchi TP, Conlon RA, Rossant J, Breitman ML (1992): tek, a novel tyrosine kinase gene located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors. Oncogene 7:1471–1480.

Dumont DJ, Gradwohl GJ, Fong G-H, Auerbach R, Breitman ML (1993): The endothelial-specific receptor tyrosine kinase, tek, is a member of a new subfamily of receptors. Oncogene 8:1293–1301.

Fantl WF, Johnson DE, Wiliams LT (1993): Signalling by receptor tyrosine kinases. Ann Rev Biochem 62:453–481.

Firmbach-Kraft I, Byers M, Shows T, Dalla-Favera R, Krolewski JJ (1990): tyk 2, a novel class of non-receptor tyrosine kinase genes. Oncogene 5:1329–1336.

Karn T, Holtrich U, Brauninger A, Bohme B, Wolf G, Rubsamen-Waigmann H, Strebhardt K (1993): Structure, expression and chromosomal mapping of TKT from man and mouse: A new subclass of receptor tyrosine kinases with a factor VIII-like domain. Oncogene 8:3443–3440.

Miska D, Bosmann HB (1980): Existence of an upper limit to elongation of the prereplicative period in confluent cultures of C3H10T1/2 cells. Biochem Biophys Res Commun 93:1140–1145.

Moxham C, Jacobs S (1992): Insulin-like growth factors receptors. In Schoheld PN (ed): "Insulin-Like Growth Factors." Oxford Univ. Press, New York pp 80–109.

Muller M, Briscoe J, Laxton C, Guschin D, Ziemiecki A, Silvennoinen O, Harpur AG, Barbieri G, Witthuhn BA, Schindler C, Pellegrini S, Wilks AF, Ihle JN, Stark GR, Kerr IM (1993): The protein tyrosine kinase JAK1 complements defects in interferon-alfa/beta and -gama signal transduction. Nature 366:129–135.

O'Bryan JP, Frye RA, Cogswell PC, Neubauer A, Kitch B, Prokop C, Espinoza R III, Le Beau MM, Shelton Earp H, Liu ET (1991): axl, a transforming gene isolated from primary human myeloid leukemia cells, encodes a novel receptor tyrosine kinase. Mol Cell Biol 11:5016–5031.

Owen TA, Cosenza SC, Soprano DR, Soprano KJ (1987): Time of c-fos and c-myc expression in human diploid fibroblasts stimulated to proliferate after prolonged quiescence. J Biol Chem 262:15111–15117.

Owen TA, Soprano DR, Soprano KJ (1989): Analysis of the growth factor requirements of WI-38 cells after extended periods of density-dependent growth arrest. J Cell Physiol 139:424–431.

Owen TA, Carter R, Whitman MW, Soprano DR, Soprano KJ (1990): Evidence that density-dependent growth arrest is a two-stage process. J Cell Physiol 142:137–148.

Peña A, Reddy CD, Wu S, Hickok N, Reddy EP, Yumet G, Soprano DR, Soprano KJ (1993): Regulation of human ornithine decarboxylase expression by the c-myc/max protein complex. J Biol Chem 268:27277–27285.

Pietrzkowski Z, Lammers R, Carpenter G, Soderquist AM, Limardo M, Philips PD, Ulrich A, Baserga R (1992): Constitutive expression of insulin-like growth factor 1 and insulin-like growth factor 1 receptor abrogates all requirements for exogenous growth factors. Cell Growth Differ 3:199–205.

Reiss K, Porcu P, Sell C, Pietrzkowski Z, Baserga R (1992): The insulin-like growth factor receptor is required for the proliferation of hemopoietic cells. Oncogene 7:2243–2248.

Sambrook J, Fritsch EF, Maniatis T (1989): "Molecular Cloning." 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Press.

Sawyers CL, McLaughlin J, Goga A, Havlik M, Witte O (1994): The nuclear tyrosine kinase c-abl negatively regulates cell growth. Cell 77:121–131.

Sell C, Ptasznik A, Chang C-D, Swantek J, Cristofalo VJ, Baserga R (1993): IGF-1 receptor levels of young and senescent fibroblasts. Biochem Biophys Res Commun 194: 259–265.

Sell C, Dumenil G, Deveaud C, Miura M, Coppola D, DeAngelis T, Rubin R, Efstratiadis A, Baserga R (1994): Effect of null mutation of the insulin-like growth factor 1 receptor gene on growth and transformation of mouse embryo fibroblasts. Mol Cell Biol 14:3604–3612.

Soprano KJ (1994): WI-38 cell long-term quiescence model system: A valuable tool to study molecular events that regulate growth. J Cell Biochem 54:405–414.

Toscani A, Cosenza SC, Owen TA, Soprano DR, Soprano KJ (1987): Normalization of multiple RNA samples using an in vitro synthesized external standard cRNA. Anal Biochem 165:309–319.

Welch PJ, Wang JYJ (1993): A C-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-abl tyrosine kinase in cell cycle. Cell 75:779–790.

Wilks AF (1989): Two putative protein-tyrosine kinases identified by application of the polymerase chain reaction. Proc Natl Acad Sci USA 86:1603–1607.

Wilks AF (1991): Cloning members of protein-tyrosine kinase family using polymerase chain reaction. Methods Enzymol 200:533–546.

Wilks AF, Harpur AG, Kurban RR, Ralph SJ, Zurcher G, Ziemiecki A (1991): Two novel protein-tyrosine kinases, each with a second phosphotransferase-related domain, define a new class of protein kinase. Mol Cell Biol 11:2057–2065.

Yamanashi Y, Fukushige S-I, Semba K, Sukegawa J, Nobuyuki M, Matsubara K-I, Yamamoto T, Toyoshima K (1987): The yes-related cellular gene lyn encodes a possible tyrosine kinase similar to $p56^{lck}$. Mol Cell Biol 7:237–243.

Yoshinouchi M, Baserga R (1993): The role of the IGF-1 receptor in the stimulation of cells by short pulses of growth factors. Cell Prolif 26:139–146.