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# EGF RECEPTOR DELETIONS DEFINE A REGION SPECIFICALLY MEDIATING STAT TRANSCRIPTION FACTOR ACTIVATION

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**Binding of EGF to its cognate receptor results in receptor-dimerisation, autophosphorylation and activation of intracellular signal transduction pathways. Autophosphorylated tyrosine residues in the receptor complex bind to SH2-domain containing signalling molecules and these are then often themselves phosphorylated by the receptor kinase. A critical role for these SH2-binding sites, however, is unclear. We have investigated the stimulation of (SH2-domain containing) STAT transcription factor activity, in comparison with MAP kinase activity, in cell lines expressing EGF receptor deletion mutatants. Data indicate that two autophosphorylated tyrosine residues Y1068 and Y1086 are critical for STAT activation in contrast to MAP kinase activation. Significantly, these tyrosine residues conform to a consensus YXXQ binding site and suggest direct binding of STAT-proteins to the EGF receptor.** 

The binding of epidermal growth factor to its cognate receptor at the plasma membrane results in activation of receptor tyrosine kinase, phosphorylation of intracellular substrates and finally proliferation (1). Autophosphorylation of the carboxyterminus of the receptor provides potential binding sites for several SH2 domain containing proteins (2,3). For example, the adaptor molecule GRB2 binds to the autophosphorylated EGF receptor through its SH2 domain and the p21ras guanine nucleotide release factor, Son of Sevenless (SOS), to GRB2 through its SH3 domain (4). Formation of this complex is then linked to p21ras signalling and the stimulation of a protein kinase cascade that results in MAP kinase activation and the phosphorylation and activation of transcription factor activities (5).

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**Abbreviations :** EGF, epidermal growth factor; ERK, extracellular regulated kinase; STAT, signal transducer and activator of transcription; ICAM, intercellular adhesion molecule; JAK, Janus Kinase.

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However, the requirement for EGF receptor autophosphorylation sites in mediating proliferative processes is still not clear.

Very recently a new family of tyrosine phosphorylated transcription factors has been reported termed STATs, for Signal Transducers and Activators of Transcription (6,7). These factors were uncovered through study of the transcriptional response to interferons but now appear to be regulated by a plethora of cytokines and growth factors. Receptor activation results in tyrosine phosphorylation and activation of members of the Janus (JAK) family of protein kinases, STAT tyrosine phosphorylation and translocation to the nucleus where the phosphorylated STAT proteins bind specific DNA elements activating transcription. Stimulation of the EGF receptor also results in tyrosine phosphorylation and activation of at least two STAT family members: Stat1$\alpha$ and Stat3, which can form both homo- and heterodimers, a well as the activation of JAK1 (8-13). This has been demonstrated in cultured cells and more recently in mouse liver after intraperitoneal injection of EGF (14). In this study we have analysed the activation of STAT transcription factors by EGF in cell lines containing wild-type and mutant EGF receptors. In contrast to MAP kinase activation, two critical autophosphorylated tyrosine residues have been identified mediating EGF activation of STATs. These autophosphorylation sites both contain the motif YXXQ which probably mediates direct binding of STATs to the EGF receptor. This suggests a mechanism by which there is direct phosphorylation of transcription factor by EGF receptor kinase activity.

## MATERIALS AND METHODS

*Cell lines and antibodies.* A431 cells were purchased from ATCC while EGF receptor stable cell lines were a kind gift of Dr. J. Schlessinger and have been previously described (15). All cell lines were cultured in monolayer in 6cm$\phi$ tissue culture dishes with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal calf serum (FCS), penicillin-streptomycin solution (10,000 IU/ml penicillin and 10mg/ml streptomycin), and L-glutamate (Gibco). Cells were trypsinised and plated at 30% confluency. 16 hours prior to stimulation media was changed to DMEM supplemented with 0.5% FCS. The anti-ERK2 polyclonal sera was a kind gift of Dr. J.L. Bos, Utrecht and has been previously described (16).

*Gel retardation assays.* Nuclear extracts were prepared from unstimulated and stimulated cells following a previously described procedure (17). A pIRE(IC) oligonulceotide 5'-agcttAGGTTTCCGGGAAAGCAc-3' was synthesised and double-stranded oligonucleotides were prepared by annealing the complementary strand. Oligonucleotides were labelled by filling in cohesive ends with [$\alpha^{32}$P]dCTP using Klenow fragment of DNA polymerase I. DNA fragments were seperated from unincorporated oligonucleotides by polyacrylamide gel electrophoresis. Gel retardation assays were carried out according to previously published procedures with slight modification (18). Briefly, 5$\mu$g nuclear extract was incubated in a final volume of 20$\mu$l, containing 10mM HEPES pH 7.8, 50mM KCl, 1mM EDTA, 5mM $MgCl_2$, 10% (v/v) glycerol, 5mM dithiothreitol, 2$\mu$g poly(dI-dC) and 20$\mu$g bovine serum albumin with 0.1-1.0ng of $^{32}$P-labelled pIRE(IC) oligonucleotide for 20 minutes at room temperature. Subsequently, samples were run for 2 hours on a 5% non-denaturing polyacrylamide gel at room temperature, vacuum dried and exposed to Fuji RX film at -70°C for 1-2 days.

*ERK2 activation.* Cells were cultured as described above and treated with 50ng/ml EGF for 10 minutes. After treatment, cells were lysed in Laemmli buffer essentially as described before (16). After being heated to 95°C for 5 minutes, total cell lysates were run out on a 12.5% SDS-polyacrylamide gel. Proteins were transferred to Immobilon-P and blots were incubated with polyclonal ERK2 antibody. Detection was with horseradish peroxidase coupled to secondary antibody utilising enhanced chemiluminescence (Amersham).

## RESULTS

Previous studies have demonstrated the activation of STAT transcription factors by EGF treatment of responsive cells, and in mouse liver after intraperitoneal injection of this growth factor (8-13). The mechanism by which EGF treatment causes tyrosine phosphorylation of STAT transcription factors is not known although it is probably independent of p21ras (11). EGF treatment also results in the tyrosine phosphorylation of JAK1 (12) so it could be that the EGF receptor does not directly phosphorylate STATs but does so via activation of JAK1. To define further the role of the EGF receptor in activation of STAT family members we took advantage of a series of stable cell lines that overexpress various receptor deletions (15). HER14 is an NIH3T3 stable cell line expressing 3.0 x $10^5$ human EGF receptors. Derivatives of this cell line are shown in Figure 1 and include : ΔINT, lacking the complete intracellular domain of the receptor; K721A, a kinase-dead receptor that is no longer catalytically active; Δ63, lacking the last 63 amino acids; and Δ126, lacking the last 126 amino acids (Fig. 1).

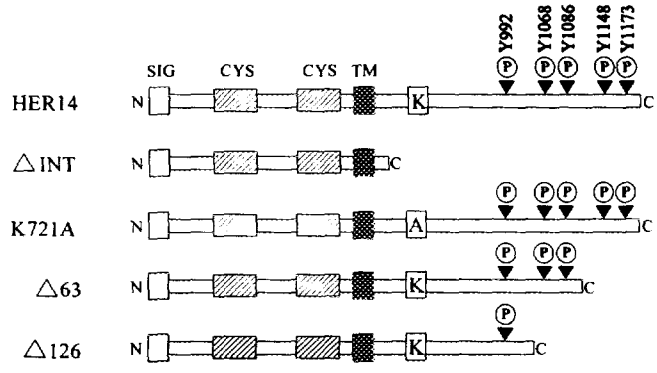


**Figure 1. Schematic representation of wild-type and mutant EGF receptors.** The following abbreviations are used: *SIG*, signal sequence; *CYS*, the two cysteine region domains; *TM*, transmembrane domain; *HER14*, wild-type human EGF receptor; *ΔINT*, EGF receptor with deletion of the entire intracellular domain; *K721A*, kinase-dead EGF receptor containing lysine to alanine mutation in catalytic domain; *Δ63*, EGF receptor with a 63-amino acid deletion from the C-terminus; and *Δ126*, EGF receptor with a 126-amino acid deletion from the C-terminus. The positions of autophosphorylated tyrosine residues are indicated.

To measure STAT activation we performed gel retardation assays utilising a pIRE probe that we had previously defined as a STAT binding site in the ICAM-1 promoter (19). Cells were starved for 16 hours before stimulating with 50ng/ml EGF and collecting samples for nuclear extract preparation at the times indicated. The first panel shows EGF-induced STAT-binding activity in A431 (Fig.1). Three complexes are marked corresponding to A, STAT3 homodimers; B, STAT1$\alpha$/STAT3 heterodimers; and C, STAT1$\alpha$ homodimers (13). These cells express 6.0 x $10^5$ EGF receptors and there is a long-term activation of STAT-binding lasting for more than 4 hours. HER14 cells expressing less receptors show a more transient STAT activation, returning to basal levels in less than 4 hours, but with the same three DNA-binding complexes formed. As might be expected, the ΔINT cell line which lacks the intracellular portion of the receptor, is unable to activate STAT DNA-binding (see third panel). Similarly, the kinase-dead K721A NIH3T3 cell line is also unable to activate STAT DNA-binding. The Δ63 deletion mutant was still able to activate STAT DNA-binding to the same level as that observed in the wild-type HER14 cells. This suggests that the two C-terminal autophosphorylation sites (Y1148 and Y1173) are not required for EGF receptor mediated STAT-activation. Most interstingly, however, the Δ126 mutant lacking also the Y1068 and Y1086 autophosphorylation sites, was no longer active in respect to STAT activation after the addition of EGF.

To determine if the mechanism of activation of STAT transcription factors by EGF correlates with the well defined activation of p21ras signal transduction we analysed the above receptor mutants for activation of MAP kinase (ERK2). Cells were again starved for 16 hours prior to stimulation with 50ng/ml EGF for 5 minutes (Fig.3). The activation of MAP kinase can be measured by a "shift" on SDS-polyacrylamide gels due to the retarded mobility of phosphorylated ERK2 (16). As can be seen from Figure 3, MAP kinase is strongly activated in the wild-type HER14 cells. Again as might be expected, deletion of the intracellular domain (ΔINT) abolishes MAP kinase activation. Furthermore, the kinase-dead EGF receptor (K721A) is also unable to activate ERK2 in this NIH3T3 cell line. Stimulation of the Δ63 cell line, lacking the last two C-terminal autophosphorylation sites, results in ERK2 activation, as does further deletion (Δ126) removing all four C-terminal sites. These data demonstrate that activation of MAP kinase does not require the prescence of EGF receptor autophosphorylation sites.

## DISCUSSION

The recognition of receptor phosphotyrosine residues by intracellular signalling molecules containing SH2 domains is a central focus for current mechanistic models of receptor tyrosine kinase signal transduction (20,21). Specificity is regulated by the sequences

surrounding a phosphotyrosyl residue which have preferential interaction with individual SH2 domain-containing molecules. Studies of the PDGF receptor have demonstrated that mutagenesis of specific autophosphorylation sites prevents binding of certain SH2-containing proteins and often results in reduced signalling capacity for this receptor (22-27). Similar studies have been performed with the EGF receptor although the results are less clear. For example, mutation of each of the autophosphorylation sites individually did not dramatically impair association with SH2-containing molecules such as phospholipase C-$\gamma$1 or the p85 subunit of phosphatidylinositol 3-kinase, as measured by co-immunoprecipitation (28). However, mutation of all autophosphorylation sites to phenylalanine does result in the inhibtion of binding of SH2 domain containing signalling intermediates to activated EGF receptor complexes (28-31). Interestingly, these mutant receptors are fully able to activate certain downstream signal transduction pathways resulting in gene expression and mitogenesis (28-31). It appears that one mechanism utilised is the use of phosphorylated cellular proteins, such as Shc, as surrogates for binding SH2 domain proteins (28,30,31).

Our study has demonstrated that in terms of STAT transcription factor activation there is a critical requirement for two of the EGF receptor autophosphorylation sites, Y1068 and Y1086 (Fig. 2). This is in direct contrast to the studies described above and to our observations of MAP kinase activation in these NIH3T3 cell derivatives (Fig. 3). Presumably MAP kinase activation indeed procedes through the tyrosine phosphorylation of other cellular proteins such as Shc, which then provide binding sites for a GRB2/SOS complex which resulting in activation of p21ras signalling to MAP kinase (28, 30,31). It appears from our data that activation of STATs is not able to proceed through such a surrogate intracellular tyrosine phosphorylated protein.

STAT transcription factors are themselves tyrosine phosphorylated and contain SH2 domains that are critical for binding to both activated cytokine receptors as well as for the formation of both homo- and hetero-dimers necessary for DNA binding (32). For most cytokine receptors, activation of STATs occurs though phosphorylation by members of the JAK tyrosine kinases associated directly with the receptor complex. Indeed, addition of EGF to A431 cells has been shown to activate JAK1 by an as yet unknown mechanism (12). It is so far unclear if the activation of STATs by EGF is due to (a) direct tyrosine phosphorylation by the EGF receptor itself or (b) is mediated via activation of JAK1. Our data suggest that autophosphorylated tyrosine residues Y1068/Y1086 are critical in one of these two processes.

During the preparation of this manuscript, Stahl *et. al.* have reportedthe identification of modular tyrosine-based motifs in gp130 cytokine receptors that mediate the specific activation of STATs (33). Receptor chimarae studies and point mutations have revealed that activation of specific STATs by IL-6 or LIF appears to be determined not by which JAK is associated

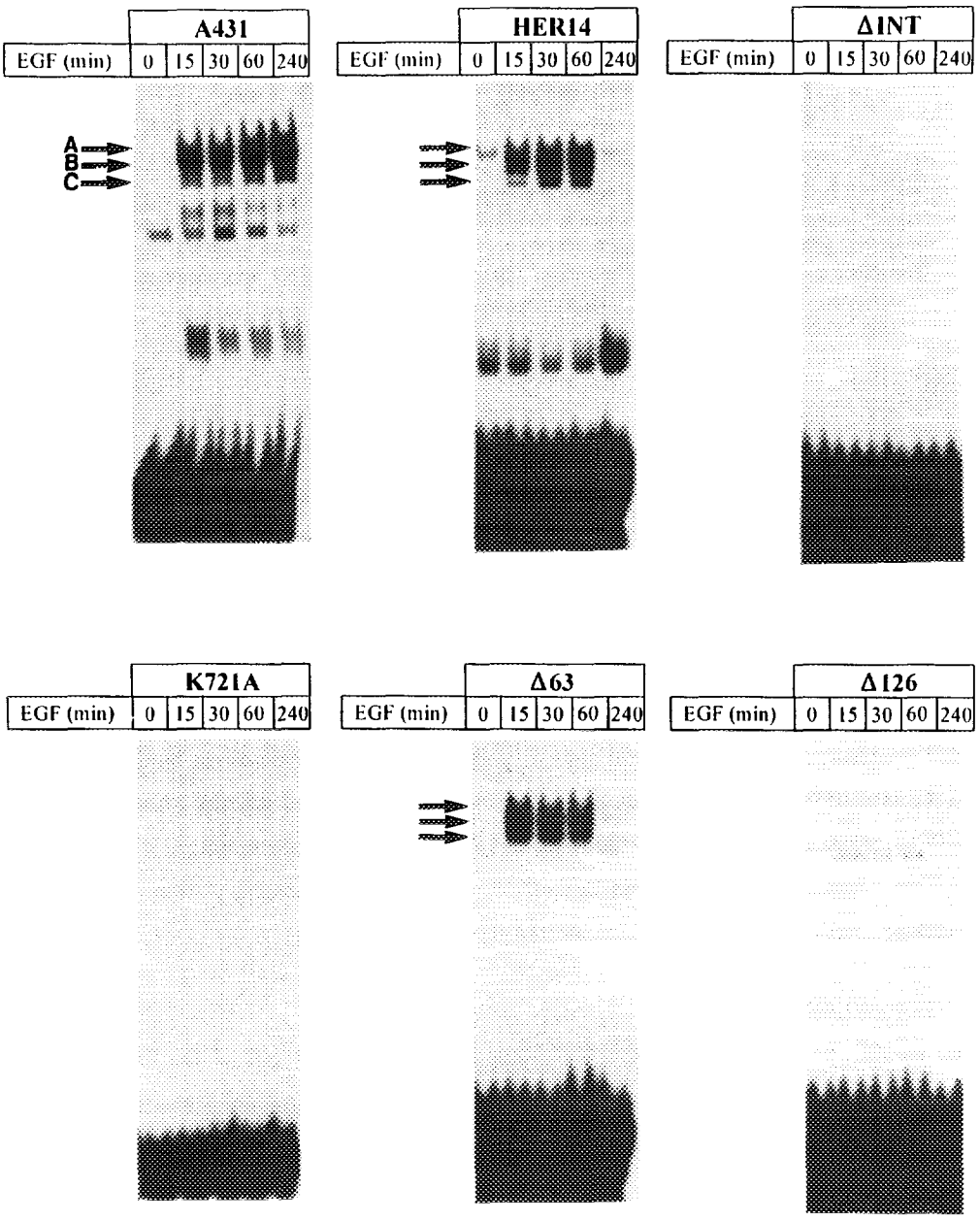


**Figure 2. Effect of EGF receptor deletion analysis on STAT activation.** NIH3T3 cells stably transfected with the human EGF receptor and mutated derivatives were either untreated or treated with 50ng/ml EGF fot the indicated times. 5$\mu$g of nuclear protein extract was mixed with double-stranded $^{32}$P-labelled ICAM-1 pIRE oligonucleotide and analysed by gel retardation analysis as described under Materials and Methods. The positions of three complexes are indicated: *A*, Stat3/Stat3 homodimer; *B*, Stat1$\alpha$/Stat3 hetrodimer; and *C*, Stat1$\alpha$/Stat1$\alpha$ homodimer.

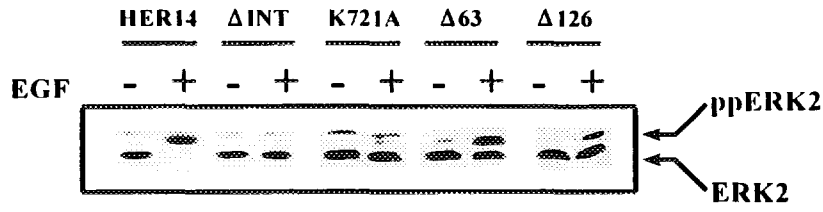


**Figure 3. EGF receptor autophosphorylation sites are not required for ERK2 activation.** NIH3T3 cells stably transfected with the human EGF receptor and mutated derivatives were either untreated (-) or treated (+) with 50ng/ml EGF for 10 minutes. Total cell lysates were prepared and analysed after SDS-PAGE by Western blotting with αERK2 antisera as described under Materials and Methods. The modification of ERK2 was measured by the occurrence of a mobility shift (indicated by an arrow, ppERK2).

to a cytokine receptor complex, but instead, by the specific tyrosine-based motif (SH2-binding site) present in the receptor components. They conclude that the sequence YXXQ appears to be a preferential STAT binding site (33). Most interestingly, our deletion analysis has revealed a critical role for tyrosine residues Y1068 and Y1086 in the EGF receptor and both these sites conform to this consensus : $^{1068}$YINQ and $^{1086}$YHNQ. Deletion of the two C-terminal autophosphorylation sites Y1148 and Y1173 had no effect on STAT activation, and neither of these sites conform to this consensus : $^{1148}$YQQD and $^{1173}$YLRV. This suggests that EGF receptor autophosphorylation sites Y1068 and Y1086 provide direct binding sites for STAT transcription factors. Thus a model can now be proposed wherby after addition of EGF to target cells, the EGF receptor becomes autophosphorylated resulting in direct binding of STAT transcription factors via SH2-domain interactions, phosphorylation of STAT by receptor kinase activity and translocation to the nucleus.

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## REFERENCES

1. Schlessinger, J., and Ulrich, A. (1992) Neuron 9, 383-391
2. Pawson, T. and Gish, G.D. (1992) Cell 71, 359-362
3. Mayer, B.J. and Baltimore, D. (1993) Trends Cell Biol. 3, 8-13
4. Pawson, T. and Schlessinger, J. (1993) Curr. Biol. 33, 434-442
5. Hill, C.S. and Treisman, R. (1995) Cell 80, 199-211
6. Darnell, J.E., Kerr, I.M. and Stark, G.R. (1994) Science 264, 1415-1421
7. Ihle, J.N., Witthuhn, B.A., Quelle, F.W., Yamamoto, K., Thierfelder, W.E., Kreider, B. and Silennoinen, O. (1994) Trends Biochem. Sci. 19, 222-227

8. Larner, A.C., David, M., Feldman, G.M., Igarashi, K., Hackett, R.H., Webb, D.S.A., Sweitzer, S.M., Petricoin III, E.F. and Finbloom, D.S. (1993) Science 261, 1730-1733
9. Sadowski, H.B., Shuai, K., Darnell, J.E. and Gilman, M.Z. (1993) Science 261, 1739-1744
10. Ruff-Jamison, S., Chen, K. and Cohen, S. (1993) Science 261, 1733-1736
11. Silvennoinen, O., Schindler, C., Schlessinger, J. and Levy, D.E. (1993) Science 261, 1736-1739
12. Shuai, K., Ziemiecki, A., Wilks, A.F., Harpur, A.G., Sadowski, H.B., Gilman, M.Z. and Darnell, J.E. (1993) Nature 366, 580-582
13. Zhong, Z., Wen, Z. and Darnell, J.E. (1994) Science 264, 95-98
14. Ruff-Jamison, S., Zhong, Z., Wen, Z., Chen, K., Darnell, J.E. and Cohen, S. (1994) J. Biol. Chem. 269, 21933-21935
15. Margolis, B.L., Lax, I., Kris, R., Dombalagian, M., Honegger, A.M., Howk, R., Givol, D., Ullrich, A. and Schlessinger, J. (1989) J. Biol. Chem. 264, 10667-10671
16. de Vries-Smits, A.M., Burgering, B.M.T., Leevers, S.J., Marshall, C.J. and Bos, J.L. (1992) Nature 357, 602-604
17. Andrews, N.C. and Faller, D.V. (1991) Nucleic Acids Res. 19, 2499
18. Fried, M. and Crothers, D.M. (1981) Nucleic Acids Res. 9, 6505-6525
19. Caldenhoven, E., Coffer, P., Yuan, J., Van De Stolpe, A., Horn, F., Kruijer, W. and Van Der Saag, P.T. (1994) J. Biol. Chem. 269, 21146-21154
20. Pawson, T. (1995) Nature 373, 573-580
21. Cohen, G.B., Ren, R. and Baltimore, D. (1995) Cell 80, 237-248
22. Ronnstrand, L., Mori, S., Arridson, A.K., Erikson, A., Wernstedt, C., Hellman, U., Claesson-Welsh, L. and Heldin, C.H. (1992) EMBO J. 11, 3911-3919
23. Valius, M., Bazenet, C. and Kazlauskas, A. (1993) Mol. Cell. Biol. 13, 133-143
24. Kazlauskas, A., Kashishian, A., Cooper, J.A. and Valius, M. (1992) Mol. Cell. Biol. 12, 2534-2544
25. Fantl, W.J., Escobedo, J.A., Martin, G.A., Turck, C.W., Del Rosario, M., McCormick, F. and Williams, L.T. (1992) Cell 69, 413-423
26. Kashishian, A., Kazlauskas, A. and Cooper, J.A. (1992) EMBO J. 11, 1373-1382
27. Nishimura, R., Li, W., Kashishian, A., Mondino, A., Zhou, M., Cooper, J. and Schlessinger, J. (1993) Mol. Cell. Biol. 13, 6889-6896
28. Soler, C., Alvarez, C.V., Beguinot, L. and Carpenter, G. (1994) Oncogene 9, 2207-2215
29. Decker, S. (1993) J. Biol. Chem. 268, 9176-9179
30. Li, N., Schlessinger, J. and Margolis, B. (1994) Oncogene 9, 3547-3465
31. Gotoh, N., Tojo, A., Muroya, K., Hashimoto, Y., Hattori, S., Nakamura, S., Takenawa, T., Yazaki, Y. and Shibuya, M. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 167-171
32. Ihle, J.N. and Kerr, I.A. (1995) Trends Biol. Sci. 11, 69-74
33. Stahl, N., Farruggella, T., Boulton, T.G., Zhong, Z., Darnell, J.E. and Yancopolous, G.D. (1995) Science 267, 1349-1353