Identification of ErbB3-stimulated genes using modified representational difference analysis

Carl F. EDMAN*, Sally A. PRIGENT*†, Andrea SCHIPPER and James R. FERAMISCO

Cancer Center, University of California, San Diego School of Medicine, La Jolla, CA 92093-0684, U.S.A.

The epidermal growth factor receptor (EGFR) family of tyrosine kinases is involved in the growth of normal and tumour cells. The specific contribution of each of the four family members to these processes remains unclear. In the present study we have used a PCR-based subtractive approach to identify differences in messages induced in response to activation of ErbB3 and EGFR. The approach described is a modification of the representational difference analysis technique adapted for analysis of cDNA, which we have modified to permit identification of differential gene expression using as little as 20 μg of total RNA as the starting material. The mRNA obtained from EGF-stimulated NIH-3T3 cells expressing chimaeric EGFR–ErbB3 receptors provided the tester amplicons (small PCR-amplified fragments) which were subtracted against driver amplicons derived from unstimulated NIH-3T3 cells expressing the EGFR–ErbB3 chimaera or EGF-stimulated NIH-3T3 cells overexpressing the EGFR. A total of 22 different clones were isolated, 90% of which showed increased expression in the tester amplicons. Six of these, corresponding to known DNA sequences, were selected for further Northern blot analysis against total RNA prepared from the starting cell lines. Of these, the gene encoding the protein dlk (or a closely related protein, Pref-1) was identified as being regulated by ErbB3 but not by the EGFR. Other genes appeared to be elevated by both ErbB3 and EGFR, including those encoding c-jun, Ret finger protein (RFP), neuroleukin and amyloid protein precursor. One gene product, TIS11, was identified as being regulated by EGFR but not by ErbB3.

INTRODUCTION

ErbB3 is one of four known members of the epidermal growth factor (EGF) family of receptor tyrosine kinases [1,2]. These receptors are involved in normal cellular growth and differentiation, and have been implicated in a variety of human cancers [3,4]. At least 15 EGF-like ligands have now been identified which form a complex series of interactions with different members of the EGF receptor (EGFR) family that promote either homo- or hetero-dimerization [5,6]. Since different members of the EGFR family are able to interact with different combinations of downstream effectors, and different ligands are able to activate different combinations of receptor pairs, the system offers the potential for enormous signal diversification. Ultimately the activation of different effector pathways downstream of the receptors would be expected to result in the transcriptional activation of distinct genes. Although many studies have looked at similarities and differences in effector pathways utilized [7–9], no studies have explored the consequences of activation of different receptors at the level of gene transcription.

ErbB3 was the third member of the EGFR family to be identified, and its biology has been less extensively studied than that of the EGFR and ErbB2. ErbB3 is widely distributed in normal cell types and is elevated in certain tumours [10–15]. It is the least related to other members of the EGFR family, and contains some unusual features within the kinase domain [2]. ErbB3 possesses little or no endogenous kinase activity [16], but it is phosphorylated in response to the binding of heregulin/*neu* differentiation factor/acetylcholine receptor inducing activity when present either in homodimers [17] or in heterodimers with ErbB2 or ErbB4 [18–20]. In order to investigate signalling events activated by ErbB3, we [21] and others [7,22] have generated cell lines expressing chimaeric receptors containing the extracellular domain of the EGFR linked to the intracellular domain of ErbB3 (ErbB3*). This facilitates the convenient phosphorylation of ErbB3 by EGF, and the recruitment of downstream effectors. Using this system we have demonstrated that, in contrast with the EGFR, ErbB3 contains many potential binding sites for the p85 subunit of phosphatidyl inositol 3′-kinase [7,21]. One might expect heterodimeric complexes containing ErbB3 to be more potent activators of phosphoinositide 3-kinase than are EGFR homodimers, and indeed the EGFR has been shown to activate phosphoinositide 3-kinase through heterodimerization with ErbB3 [23].

In the present study we were interested in determining whether we could delineate differences in gene expression in response to activation of ErbB3 relative to that of the EGFR. Advantage was taken of a PCR-based subtractive hybridization technique, representational difference analysis (RDA), which was developed by Wigler and colleagues [24] and adapted by ourselves and others [25–27] for cDNA (cDNA-RDA). Using this approach, we have successfully isolated cDNA fragments corresponding to mRNAs known to be induced by growth factors (e.g. that for c-jun), in addition to fragments encoding less well characterized, but potentially interesting, regulatory proteins.

EXPERIMENTAL

Cell culture and cDNA construction

Cell lines expressing either EGFRs or ErbB3* receptors [21] and parental NIH-3T3 cells were grown in Dulbecco's modified

Abbreviations used: EGF, epidermal growth factor; EGFR, EGF receptor; ErbB3*, chimaeric ErbB3 receptor; +EGFR, EGF-stimulated EGFR; −EGFR, unstimulated EGFR; +ErbB3*, EGF-stimulated ErbB3*; −ErbB3*, unstimulated ErbB3*; oligo, oligonucleotide; RDA, representational difference analysis; RDA-cDNA, RDA adapted for the analysis of cDNA; RFP, Ret finger protein; TE, 1 mM EDTA/10 mM Tris/HCl, pH 8.0.

* These authors contributed equally to this work.

† To whom correspondence should be sent, at present address: Department of Biochemistry, University of Leicester, Adrian Building, University Road, Leicester LE1 7RH, U.K.

Table 1 Oligos used for cDNA-RDA (*Msp*I site)

Oligo no.	Sequence (5′–3′)
1	TGCACTCTCCAGCCTCTCACCGAC
2	CGGTCGGTGA
3	TCCAGCGTCGTCTATCCATGAACC
4	CGGGTTCATG
5	TGCGAACTGTGCTATCCGAGGGAC
6	CGGTCCCTCG

Eagle's medium containing 10 % (v/v) fetal bovine serum at 37 °C in an atmosphere of 10 % CO_2. Cells at 70 % confluency were starved in low-serum medium (0.5 %) for 24 h prior to a 2 h stimulation with EGF (20 pM). Cells were washed with PBS, and total RNA was isolated using RNeasy Total RNA kits (Qiagen). Double-stranded cDNA was synthesized from mRNA isolated from 20–40 μg of total RNA using Oligotex mRNA (Qiagen) and RiboClone cDNA Synthesis (Promega) kits. Double-stranded cDNA was purified using a Qiagen QIAquick-spin PCR purification kit and eluted in 40 μl of water. All kits were utilized according to the manufacturer's instructions.

Modified RDA using cDNA

The original protocol [24] was used with the following modifications. All double-stranded cDNA was digested with *Msp*I (Gibco-BRL), and oligonucleotides (oligos) 1 and 2 (Table 1) were ligated on to the ends using 500 pmol of each oligo, 6.6 μl of 5 × T4 DNA ligase buffer (Gibco-BRL), 15 mM NaCl and water in a final volume of 34 μl. After ligation overnight at 12 °C, the mixtures were diluted with 66 μl of TE containing 20 μg/ml tRNA. Amplicons were generated in replicate PCR reactions consisting of 5 μl of the diluted ligation mixture, 124 pmol of oligo 1, 10 μl of 10 × PCR buffer (Boehringer-Mannheim), 320 μM each of dATP, dCTP, dGTP and dTTP, and water in a final volume of 100 μl, using 5 units of *Taq* DNA polymerase (Boehringer-Mannheim) and 20 cycles of amplification. Products from these PCR reactions and all subsequent steps were isolated using QIAquick-spin PCR purification kits (Qiagen) using one column per reaction and elution with 50 μl of TE. Replicates were pooled. Concentrations were estimated using ethidium bromide [28], and typically ranged between 50 and 100 ng/μl.

The amplicons were then digested with *Msp*I, generating the driver amplicons. The construction of tester amplicons was performed with digested +ErbB3* (EGF-stimulated ErbB3*) amplicons (500 ng) ligated to oligos 3 and 4 using the ligation protocol described above. After overnight ligation, the tester amplicons were diluted with 66 μl of TE containing tRNA (20 μg/ml).

The first round of subtraction/hybridization used 50 ng of tester amplicons mixed with 5 μg of driver amplicons [either −ErbB3* (unstimulated ErbB3*) or +EGFR (EGF-stimulated EGFR) amplicons]. The mixtures were heated for 5 min at 95 °C, chilled on ice for 5 min and then precipitated using 2.5 M ammonium acetate (final concn.) followed by 3 vol. of ethanol. Hybridization was then carried out in 4 μl of 3 × EE buffer {30 mM Epps (*N*-[2-hydroxyethyl]piperazine-*N*′-[3-propanesulphonic acid])/3 mM EDTA, pH 8.0} overnight, including melting and NaCl addition, as described by Lisitsyn et al. [24]. After 20 h at 67 °C, the oil overlay was removed and 8 μl of tRNA (5 mg/ml in TE) was added, followed by 88 μl of TE.

The hybridization mixtures were amplified in three successive stages, with a mung-bean nuclease digestion step between the first and second stages. Each reaction was set up in duplicate. The first PCR amplification used 10 μl of each tester/driver hybridization mixture and PCR reactants as before (without added oligo). After preheating (3 min at 72 °C), 5 units of *Taq* DNA polymerase was added. Following an additional 5 min at 72 °C, 128 pmol of oligo 3 was added, followed by an oil overlay. Amplification consisted of 10 cycles of 1 min at 94 °C and 3 min at 72 °C. A 10 min extension at 72 °C ended the amplification. Following isolation, the products of duplicate reactions were pooled.

A mung-bean nuclease (50 units; USB) digestion used 40 μl of the PCR product in a total volume of 80 μl (30 min at 30 °C) and was terminated by adding 120 μl of 50 mM Tris/HCl, pH 8.9, and then heating (5 min at 95 °C). The second amplification used 10 μl of the mung-bean-nuclease-digested material and the same conditions employed in the initial generation of amplicons with oligo 3. After isolation, the third PCR amplification was performed in a fashion identical to the second using 1 μl of the second amplification product as the amplification template. At the end of the third PCR amplification, the products were isolated and those from duplicate reactions pooled.

After digesting the amplified product of the first round with *Msp*I, oligos 5 and 6 were ligated on to 500 ng of this digested DNA. A 6.25 ng portion of this ligated material was then mixed with 5 μg of the appropriate driver amplicons. The second round of subtraction/hybridization followed by PCR amplification was then carried out using the same conditions as for the first round described above.

Subcloning and Southern blot analysis

*Msp*I-digested RDA fragments from the first round of RDA were subcloned into the *Acc*I site of pBluescript KSII− (Stratagene). Following transformation into DH5α, individual colonies were selected, grown as overnight cultures and screened for inserts by PCR using M13 primers. Clones containing differently sized inserts were selected. Amplicon blots were constructed using 500 ng of the original amplicons (digested with *Msp*I), electrophoresed using 2 % agarose gels in the presence of 1 × Tris/borate/EDTA and transferred by capillary action to nylon membranes using standard techniques [28,29]. Insert regions were then utilized as templates for probe construction using the Ready-To-Go DNA random prime labelling kit (Pharmacia). Southern blot hybridization was performed as described [30] using a 3 h hybridization period in a volume of 5 ml.

Northern blot analysis

Total RNA (3 μg or 6 μg per lane) from NIH-3T3 cells, ErbB3* cells or EGFR cells in the presence or absence of EGF stimulation was electrophoresed using 1.2 % agarose gels containing formaldehyde and then transferred to nylon membranes as above. Probes were constructed using either random prime methodology or, for fragments of less than 400 bp in length, a PCR-based methodology [developed by Dr. Frank Furnari (personal communication)]. PCR amplification mixtures contained 1 ng of PCR-generated template, 2.5 pmol each of SK and T3 oligo primers, 1 μl of 10 × *Taq* reaction buffer (Boehringer-Mannheim), 8.25 pmol of each of dATP, dGTP and dTTP, 25 μCi of [α-^{32}P]dCTP (3000 Ci/mmol) and 1.25 units of *Taq* DNA polymerase in a total volume of 10 μl. Amplification conditions consisted of an initial melting step of 4 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C. A final extension step of 7 min at 72 °C completed the

amplification. Unincorporated nucleotides and primers were removed by G-50 column (Boehringer-Mannheim) purification. Hybridization and wash conditions were as described [30].

DNA sequence analysis

Inserts were sequenced using the Sequenase version II DNA sequencing kit (USB, Cleveland, OH, U.S.A.) according to the manufacturer's instructions. Sequence comparisons were made with sequences in GenBank using BLAST [31]. A smallest sum probability of $P(N) \leqslant 5e\text{-}30$ was observed in all clones termed homologous to existing sequences [31].

RESULTS

A modified version of RDA adapted for cDNA was employed to isolate DNA fragments associated with ErbB3 activation. Two approaches were used to generate subtracted populations of cDNA (Figure 1). In both approaches, +ErbB3* cells provided amplicons to be used as tester amplicons (i.e. those amplicons to be examined for possible gene expression differences). The first approach, Group A, utilized amplicons arising from −ErbB3* cells as driver, i.e. these amplicons provided the subtractive material. The other approach used cDNA from +EGFR cells as driver (Group B). In contrast with Group A, the subtractive process of Group B should result in the removal of amplicons common to activated ErbB3 and EGFR, thereby increasing the likelihood of identifying transcripts specifically induced in response to ErbB3 stimulation.

As shown in Figure 2, the initial subtractive/enrichment round (lane 1) produced distinct bands for both Groups A and B. A second subtractive enrichment round yielded a less complex set of bands in both groups (lane 2). In order to ensure the greatest diversity of products, we utilized the first-round material for subsequent subcloning and analysis. From Group A, 12 clones containing different sized inserts were selected for further analysis; from Group B, 10 were selected. Each insert was Southern blotted against the starting amplicons for both groups as well as against amplicons generated from unstimulated EGFR cells. Representative blots displaying the results obtained with two of these probes are shown in Figure 3. Note that for one of these probes, c-jun, hybridization was strongest in the EGF-stimulated amplicons. For the dlk probe, hybridization was clearly strongest in the +ErbB3* lane. The hybridizations observed in the lanes corresponding to the molecular mass ladder were attributable to the presence of polylinker in the probe hybridizing to corresponding regions of the fragments generating the size standards. In all, 10 Group A clones and all Group B clones showed increased hybridization in one or both of the EGF-stimulated lanes.

The positive clones were sequenced and analysed for identity with existing GenBank sequences (Table 2). A number of clones contained multiple *Msp*I fragments, thus increasing the possible

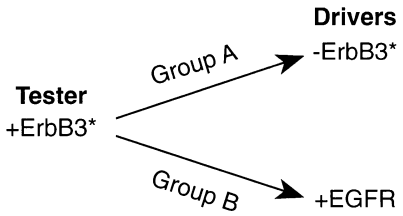

Figure 1 Tester and driver amplicon groups used in cDNA-RDA

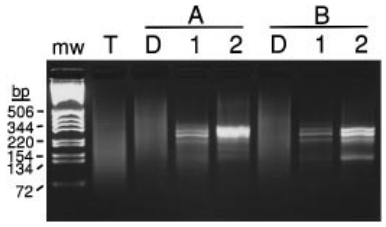

Figure 2 Gel electrophoresis of cDNA-RDA products

The products from successive stages of RDA using +ErbB3* cells as tester with either −ErbB3* cells (**A**) or +EGFR cells (**B**) as driver were separated on a 2.0% agarose gel and visualized with ethidium bromide. Lanes: mw, 1 kb ladder (Gibco-BRL); T, tester amplicon; D, driver amplicon; 1, first-round difference product; 2, second-round difference product.

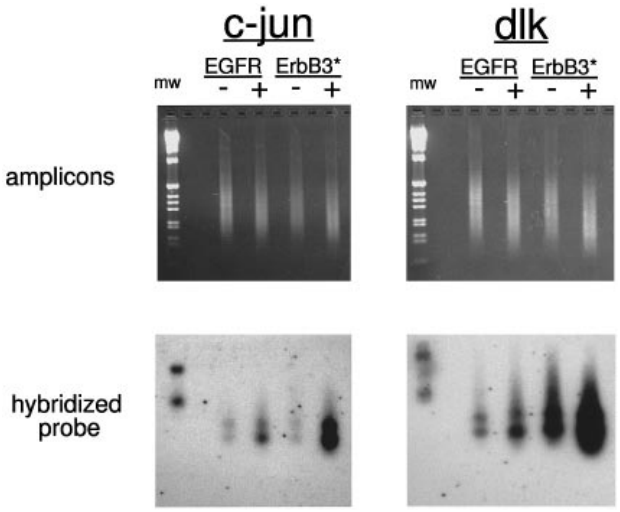

Figure 3 Southern blot analysis utilizing amplicons employed for subtractive hybridization

Amplicons generated from either EGFR or ErbB3* cells were electrophoresed on agarose gels, transferred to nylon membranes and then probed with the cloned products obtained from the first round of RDA, as described in the Experimental section. The upper panels show the ethidium bromide-stained gels for two of these probes, c-jun and dlk. The lower panels display the corresponding autoradiography results obtained with each. Lanes: mw, 1 kb ladder (Gibco-BRL); −, absence of EGF stimulation; +, presence of EGF stimulation.

Table 2 Sequences identified by cDNA-RDA

Group A	Group B
dlk	dlk
Neuroleukin	Neuroleukin
Pro-α1(I) collagen	Pro-α1(I) collagen
Pro-α2(I) collagen	Pro-α2(I) collagen
Vimentin	Vimentin
Human clone 26687	Human clone 26687
c-jun	Human ErbB3
TIS11	RFP
GEG-68	sox-4
Human clone 157865	α-Tubulin
Human clone 15B12	Human clone 53168
Amyloid β protein precursor	Human clone 61107

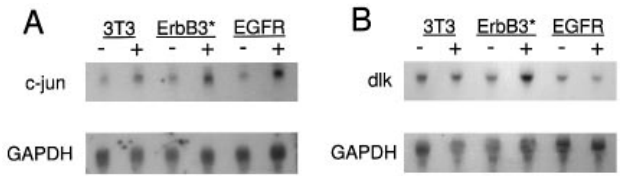

Figure 4 Northern blot analysis of NIH-3T3, ErbB3* and EGFR cells

Total RNA (3 μg per lane) was probed with either c-jun (**A**) or dlk (**B**) as described in the Experimental section. Lanes: −, absence of EGF stimulation; +, presence of EGF stimulation. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) hybridization was used as a control to determine loading and transfer efficiencies.

Table 3 Relative gene transcription as assessed by Northern blot analysis

Values are fold increases compared with levels in unstimulated parental NIH-3T3 cells, normalized for glyceraldehyde-3-phosphate dehydrogenase levels. −, Unstimulated; +, stimulated by EGF; APP, amyloid β protein precursor.

	Relative transcription					
	NIH-3T3 cells		ErbB3* cells		EGFR cells	
	−	+	−	+	−	+
TIS11	1.0	1.5	1.0	1.9	0.8	3.2
APP	1.0	1.0	1.7	3.5	2.2	2.9
Neuroleukin	1.0	1.8	4.7	5.4	3.5	3.8
RFP	1.0	1.9	3.2	5.4	3.1	3.5

number of candidate fragments available for further examination. Of the total number of *Msp*I fragments in group A, 11 of 18 showed identity to known proteins, five coded for DNA sequences generated for use as expressed sequence tags and two were unknown. In group B, 11 of 21 fragments showed identity to known proteins, three coded for expressed sequence tags and seven were unknown. Thus greater than 50% of the *Msp*I fragments were immediately identifiable as showing identity to known proteins upon GenBank comparison.

The identified *Msp*I fragments, including the expressed sequence tag fragments, are presented in Table 2. The fragments include genes encoding structural proteins (collagen [32], tubulin [33] and vimentin [34]), proteins involved in metabolism (neuroleukin [35] and glucose-6-phosphate isomerase [36]), extracellular proteins (amyloid β protein precursor [37] and dlk [38]), transcription factors {c-jun [39], TIS11 [40], Ret finger protein (RFP) [41] and sox-4 [42]}, and expressed sequence tags (the human clones and GEG-68). Of these, six fragments were found in common between the two experimental groups. We also identified, in the group using +EGFR cells as driver (Group B), a fragment encoding a portion of human ErbB3 construct used to generate the chimaeric ErbB3 cell line.

As shown in Table 2, an *Msp*I fragment of c-jun was identified among the fragments of Group A, but was not found in Group B. Since c-jun is a ubiquitous transcription factor whose expression is associated with the onset of the cell cycle, both +ErbB3* cells and +EGFR cells would be expected to respond by expressing c-jun. To establish whether c-jun expression parallels stimulation with EGF, Northern blot analysis was performed using total RNA isolated from both stimulated and unstimulated ErbB3* and EGFR cells (Figure 4A). Also included for comparison was RNA isolated from the parental 3T3 cells. Stimulation with EGF increased c-jun expression in all three cell types; however, the levels of c-jun expression in +ErbB3* cells and +EGFR cells were greater than in the stimulated parental 3T3 cells. Thus it appears that c-jun expression is elevated by stimulation of both ErbB3 and the EGFR.

In contrast to c-jun expression, which was elevated by EGF stimulation of all three cell lines, dlk expression was elevated exclusively in +ErbB3* cells (Figure 4B). This band migrated at approx. 1.6 kb, which is in accordance with the reported transcript size of dlk [38], and was increased at least 2-fold in +ErbB3* cells as compared with the other cells, as measured by the pixel densitometry of the autoradiographs. These findings were reproduced using RNA isolated from stimulated cells on two separate occasions.

Another of the identified fragments, TIS11, appears to be specifically regulated by the EGFR and not by ErbB3. As indicated in Table 3, the levels of hybridization observed in both −ErbB3* and +ErbB3* cells closely resembled the levels observed in the parental 3T3 cells. However, a 3-fold increase was observed in the +EGFR cells. Taken together, these results suggest that TIS11 expression is attributable to endogenous EGFRs in the NIH-3T3 and ErbB3* cells. The overexpression of the EGFR led to the increased levels observed in the +EGFR cells.

The results for three other fragments examined by Northern blot analysis are also presented in Table 3. Amyloid β protein precursor, neuroleukin and RFP all appeared to be up-regulated in both +ErbB3* cells and +EGFR cells. As expected, the patterns of elevation were consistent with the employment of +ErbB3* cells as tester and either −ErbB3* cells (amyloid β protein precursor) or +EGFR cells (neuroleukin, RFP) as driver. An unexpected observation was the apparent overall up-regulation of these three genes in the ErbB3* cells and EGFR cells as compared with the parental NIH-3T3 cells. It is unclear whether this response is a non-specific result of cell line construction or whether high levels of expression of these receptor types led to the elevated response.

DISCUSSION

In this paper we present a modified RDA protocol [24,26] adapted for use with cDNA and show its application in the analysis of novel gene induction by activated ErbB3* receptors. In concept, RDA has several advantages over the widely employed technique of differential display, e.g. increased likelihood of isolating fragments within open reading frames, ease of cloning and analysis, higher frequency of positive clones and possible advantages of using various driver amplicon pools, as discussed by others [25–27].

We have used an 8-fold decrease in the quantity of driver amplicons employed at each mixing/hybridization step, i.e. from 40 μg to 5 μg. This reduction is accompanied by a proportional reduction in the amount of tester amplicons, thereby maintaining the tester/driver ratio. Such a reduction correspondingly reduced the amounts of reagents, including mRNA, required and simplified the procedure. It was unclear at first whether this decrease in concentration would adversely affect the kinetics of reannealing between tester and driver amplicons during the subtraction hybridization step. However, we noted that these calculations were based upon the expected complexities of genomic DNA fragments as well as on assumptions about the expected rates of encounter between complementary DNA strands in such a

mixture [24]. Our results may reflect the lower complexity of cDNA mixtures offsetting the lower absolute concentration of DNA fragments present in the hybridization. Furthermore, the results of the Southern blot analysis indicate that the subtraction process was indeed efficient, in that the probes hybridized selectively with appropriate amplicon groups from EGF-stimulated cells. One possible caveat is that decreasing the absolute amount of starting material may lead to a reduced ability to detect low-copy-number mRNAs, and the inadvertent cloning of highly represented cDNAs in addition to induced ones. In general, however, our findings suggest that this approach may enable cDNA-RDA to be useful where only small samples of tissue are available.

Variations in the subtraction process have previously been reported by Hubank and Schatz [26], enabling removal of known genes and highly expressed genes. Their success suggested that the nature of the subtractive step is amenable to considerable manipulation. We reasoned that, by employing driver amplicons arising from EGF-stimulated cells which overexpress the EGFR, we would increase the likelihood of isolating amplicon fragments arising from ErbB3-unique mechanisms (Figure 1). This approach had some measure of success, as gauged by the recovery of a portion of the ErbB3 chimaera construct from the EGFR driver group (Table 2). The presence of dlk and several other fragments in both subtractive groups implies that the use of the EGFR driver amplicons may not have been requisite for the present study, but this observation should be considered in the light of the isolation of TIS11 while using −ErbB3* as driver (Group A). By Northern blot analysis (Table 3), TIS11 is apparently regulated by EGFR and not by ErbB3. This suggests that the use of the EGFR driver amplicons was useful as a mechanism for decreasing the likelihood of isolating such EGFR-regulated gene fragments.

In general, gene regulation by ErbB3 appears to be complex, and this in turn affected the outcome of the subtractive strategy employed. The presence of genes apparently regulated both by ErbB3* and EGFR as well as those apparently governed by ErbB3* and not EGFR suggests that ErbB3 signalling is mediated both by pathways in common with EGFR and by pathways distinct from EGFR. This is consistent with our knowledge that the EGFR and ErbB3 share some interactions, e.g. with the Shc adapter protein which is probably responsible for recruitment of the Ras pathway [21,43], and differ with respect to their interactions with other elements of key signalling pathways, e.g. phosphatidyl inositol 3′-kinase [23]. It is also possible that differences exist between these receptors at the level of gene transcription. The identification of differentially regulated transcripts permits the further study of these possibilities.

Until now, ErbB3 has been linked to the specific expression of only one gene product, the acetylcholine receptor in muscle [44]. In this instance, activation of ErbB3/ErbB2 heterodimers by heregulin is believed to induce synapse-specific transcription of acetylcholine receptor genes. The presence of ErbB3 appears to be essential for this induction due to its specific expression on end plates, whereas ErbB2 is more widely expressed throughout the surface of the myofibre membrane. Our present study has provided some potential candidates for further analysis. The gene which was most exclusively induced by ErbB3 was that encoding dlk (Delta-like) [38]. dlk is a transmembrane protein containing six EGF-like repeats in its extracellular domain and has been shown to be elevated in select neuroendocrine tumours. A cleaved, circulating form of dlk, termed fetal antigen I [45], has also been reported. The situation with dlk is somewhat confused by the existence of a closely related protein, Pref-1, whose sequence is also consistent with that of our isolated *Msp*I fragment [46]. The sequence of Pref-1 is identical to that of dlk in the extracellular domain, but some discrepancies in the DNA sequence result in a difference in the predicted amino acid sequence in the intracellular domain. For its part, Pref-1 has been shown to inhibit differentiation of preadipocytes into adipocytes [47]. We are currently raising antibodies which should distinguish between Pref-1 and dlk in order to facilitate further studies. The other gene product which showed increased induction in response to ErbB3 relative to EGFR was RFP. RFP is a putative transcription factor that is found in high levels in a number of tumour cell lines [41] and is involved in the activation of the *Ret* proto-oncogene [48]. It will be interesting to investigate the roles of these potential regulatory proteins in ErbB3 function.

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