The EMBO Journal Vol.16 No.7 pp.1610–1619, 1997

Lyn tyrosine kinase is essential for erythropoietin-induced differentiation of J2E erythroid cells

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Erythropoietin stimulates the immature erythroid J2E cell line to terminally differentiate and maintains the viability of the cells in the absence of serum. In contrast, a mutant J2E clone (J2E-NR) fails to mature in response to erythropoietin; however, it remains viable in the presence of the hormone. We have shown previously that intracellular signalling is disrupted in the J2E-NR cell line and that tyrosine phosphorylation is dramatically reduced after erythropoietin stimulation. In this study we investigated the defect in J2E-NR cells that is responsible for their inability to differentiate. Screening of numerous signalling molecules revealed that the lyn tyrosine kinase appeared to be absent from J2E-NR cells. On closer examination, both lyn mRNA and protein content were reduced >500-fold. Consistent with a defect in lyn, amphotropic retroviral infection of J2E-NR cells with lyn restored the ability of the cells to synthesize haemoglobin and enabled the cells to mature morphologically. Conversely, the ability of J2E cells to differentiate in response to epo was severely curtailed when antisense lyn oligonucleotides or a dominant negative lyn were introduced into the cells. However, erythropoietin-supported viability was unaffected by reducing lyn activity. The ability of two other erythropoietin-responsive cell lines (R11 and R24) to differentiate in response to the hormone was also reduced by dominant negative lyn. Finally, co-immunoprecipitation and yeast two-hybrid analyses indicated that lyn directly associated with the erythropoietin receptor complex. These data indicate for the first time an essential role for lyn in erythropoietin-initiated differentiation of J2E cells but not in the maintenance of cell viability.

Keywords: differentiation/erythropoietin/lyn/signalling/src family

Introduction

Lyn is a member of the src family of intracellular membrane-associated tyrosine kinases (Yamanashi *et al.*, 1987). While the amino-terminus of each kinase is unique, this family shares significant structural homology in several domains—the Src Homology (SH) 1 or kinase domain and two protein interaction domains, SH2 and SH3. Alternative splicing at the 5′ end of the *lyn* gene results in expression of p53 and p56 isoforms of the protein (Stanley *et al.*, 1991; Yamanashi *et al.*, 1991b; Yi *et al.*, 1991). Lyn is expressed mainly in haematopoetic cells of myeloid (macrophage, monocyte and platelets) and B lymphoid origin (Bolen *et al.*, 1992). It is involved in the transmission of signals from a number of receptors such as the IL-2 receptor, GM-CSF receptor, high-affinity IgE receptor (FcεRI), B-cell antigen receptor and thrombin receptor (Burkhardt *et al.*, 1991; Yamanshi *et al.*, 1991a; Campbell and Sefton, 1992; Cichowski *et al.*, 1992; Torigoe *et al.*, 1992; Corey *et al.*, 1993, 1994; Yamamoto *et al.*, 1993). Importantly, lyn has been implicated in the phosphorylation of a number of signalling molecules, including phosphoinositol-3 kinase (PI-3K), ras GTPase activating protein (GAP), phospholipase C (PLC) γ2 and mitogen-activated protein (MAP) kinase (Cichowski *et al.*, 1992; Yamanashi *et al.*, 1992; Corey *et al.*, 1993; Pleiman *et al.*, 1993). To date, there have been no links between lyn and the erythropoietin receptor (epo-R), and no suggestion of an involvement of lyn in signalling within erythroid cells.

Erythropoietin (epo) acts primarily on immature erythroid precursors to maintain viability and promote proliferation and terminal differentiation (Krantz, 1991; Jelkman, 1992; Koury and Bondurant, 1992). The epo-R and its associated protein Janus kinase 2 (JAK2) are rapidly phosphorylated (Witthuhn *et al.*, 1993; Miura *et al.*, 1994) after ligand binding. Subsequently, the Signal Transducers and Activators of Transcription (STAT) and ras/MAP kinase pathways are activated. Proteins known to be phosphorylated after epo stimulation include STAT5, Shc, $p92^{c\text{-}fes}$, PI-3K, SHP2 (Syp), ras GAP and MAP kinases. Down-regulation of the epo-R signal is achieved via association of the phosphatase SHP1 (HCP) and dephosphorylation of the receptor (Klingmuller *et al.*, 1995; Yi *et al.*, 1995).

The J2E cell line was generated by transforming immature erythroid cells with the *raf/myc*-containing J2 retrovirus (Klinken *et al.*, 1988). These cells express ~1000 epo-R per cell and proliferate and differentiate in response to epo; haemoglobin is synthesized and the cells undergo morphological alterations which culminate in a proportion of cells enucleating (Klinken *et al.*, 1988; Busfield and Klinken, 1992; Busfield *et al.*, 1993a,b,c). In addition, epo supports the viability of the cells in the absence of serum (Tilbrook *et al.*, 1996a; Chappell *et al.*, 1997). We have shown that, in J2E cells epo stimulation results in the rapid phosphorylation of epo-R and JAK2, and that JAK2 is essential for all three functions of epo, viz. proliferation, differentiation and enhanced viability (Tilbrook *et al.*, 1996b; Chappell *et al.*, 1997). In addition, STAT 5 is activated in J2E cells, and tyrosine phosphoryl-

ation of PI-3K, PLCγ, ras GAP and MAP kinase occur shortly after epo stimulation (Tilbrook *et al.*, 1996a,b).

Recently, we reported that epo signalling was disrupted in a mutant J2E clone (J2E-NR) which remains viable in the presence of epo, but does not differentiate or undergo enhanced proliferation following hormonal stimulation (Tilbrook *et al.*, 1996a). While the epo-R and its associated JAK2 molecules were tyrosine-phosphorylated in response to epo in these mutant cells, a number of other signalling molecules, including PI-3K, ras GAP and MAP kinase, were not phosphorylated. It was concluded that distinct signalling pathways existed for maturation and viability within erythroid cells and that a defect in the pathway to differentiation prevented J2E-NR cells maturing after exposure to epo.

In this study we have attempted to identify the fault in J2E-NR cells which inhibits terminal differentiation. We report here that lyn protein and mRNA were severely reduced in J2E-NR cells. Reintroduction of lyn into J2E-NR cells restored the ability of the cells to synthesize haemoglobin and differentiate morphologically in response to epo. Lyn appeared to be preassociated with the epo-R in parental J2E cells, and inhibition of lyn activity in J2E cells suppressed their capacity to differentiate, but had no effect on the ability of epo to maintain viability. Our studies demonstrate that lyn is required for differentiation of J2E cells promoted by epo, but not for enhanced viability.

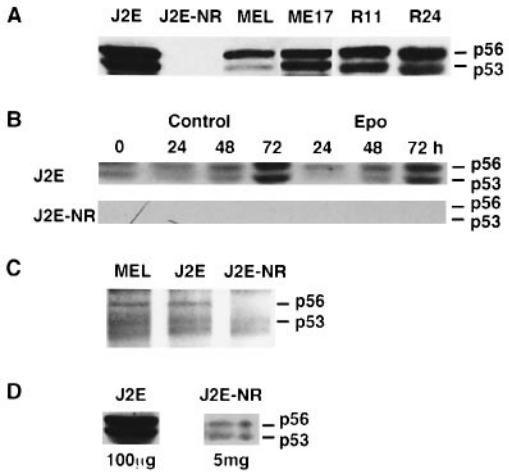

Fig. 1. J2E-NR cells express less lyn protein than J2E cells. (**A**) Immunoblot analysis of lyn in lysates from the erythroid cell lines J2E, J2E-NR, MEL, ME17, R11 and R24 (100 μg protein/lane). (**B**) Immunoblot analysis of lyn harvested over 72 h from control or epo-stimulated J2E and J2E-NR cultures (100 μg protein/lane). (**C**) SDS–PAGE analysis of lyn harvested from MEL, J2E and J2E-NR cells labelled with [^{35}S]methionine/cysteine (1.5×10^8 c.p.m.) and immunoprecipitated with rabbit anti-lyn antibodies. (**D**) Lysate (5 mg) from unstimulated J2E-NR cells, immunoprecipitated with rabbit anti-lyn antibodies and immunoblotted with anti-phosphotyrosine antibodies was compared with lysate (100 μg) from unstimulated J2E cells immunoblotted with anti-phosphotyrosine antibody. The positions of the p53 and p56 lyn proteins are indicated. The relative abundance of lyn was determined by densitometry.

Results

Substantial reduction of lyn protein in J2E-NR cells

The J2E-NR cell line was originally isolated due to its inability to proliferate or differentiate in response to epo (Klinken and Nicola, 1990). However, the cells remain alive in the presence of the hormone (Tilbrook *et al.*, 1996a). Intracellular signalling is disrupted in these cells and one of the features of this defect is a reduction in the number of molecules that are tyrosine-phosphorylated upon epo stimulation (Tilbrook *et al.*, 1996a). In an attempt to identify the defect, we screened lysates of J2E-NR cells for the presence of signalling molecules and transcription factors known to be expressed in J2E cells. Of the 27 proteins investigated (epo-R, JAK2, PLCγ, protein kinase C, PI-3K, GAP, Grb2, p52/p46 Shc, SHP-1, SHP-2, c-raf, JNK-1, MKP-1, MEK, p44/p42 MAP kinase, p90rsk, lyn, fyn, yes, src, fgr, hck, myc, jun, gata-1), only the tyrosine kinase lyn appeared to be absent from J2E-NR cells (Figure 1A and B). Other erythroid cell lines were also examined for the presence of lyn: murine erythroleukaemia (MEL) cells generated by Friend virus infection (Dube *et al.*, 1975), ME17 produced by c-*myc* retroviral infection (Cory *et al.*, 1991), and R11 and R24 which were derived by v-*raf* retroviral infection of fetal liver cells from p53–/– mice (Metz *et al.*, 1995). ME17, R11 and R24 differentiate in response to epo to varying degrees, whereas the MEL cell line does not (Cory *et al.*, 1991; Metz *et al.*, 1995; our unpublished data). All of these cell lines expressed lyn (Figure 1A).

When examined by [^{35}S]methionine/cysteine labelling of the cells, lyn was detected in lysates of J2E and MEL cells, but was undetectable in lysates of J2E-NR cells (Figure 1C). However, a small amount of tyrosine-phosphorylated lyn of the correct molecular weight could be detected in unstimulated J2E-NR cells (Figure 1D). Similar amounts of lyn were detected in J2E-NR cells after epo stimulation, but tyrosine-phosphorylated protein levels did not alter during the 60 min time-course (data not shown). When quantitated, it was found that J2E-NR cells expressed 500-fold less tyrosine-phosphorylated lyn than J2E cells. Furthermore, no lyn kinase activity could be demonstrated in J2E-NR cells (Figure 2A and C). These results demonstrate that the amount of lyn protein in J2E-NR cells is severely reduced and that no enzyme activity can be detected.

No changes in the levels of tyrosine-phosphorylated lyn were detected after epo stimulation in J2E cells (data not shown). Inactive lyn contains phosphorylated Y508 and unphosphorylated Y397, while active lyn contains dephosphorylated Y508 and phosphorylated Y397. Thus, there is no overall change in the phosphotyrosine content of lyn from being in the inactive to the active state (Sotirellis *et al.*, 1995). However, epo did induce changes in both lyn autokinase and exokinase activities (Figure 2A, B and D).

Reduced lyn mRNA in J2E-NR cells

Having observed that lyn protein levels were dramatically reduced in J2E-NR cells, we examined the structure of the *lyn* gene and production of mRNA. Southern blots of genomic DNA, digested with seven different restriction endonucleases and probed with a full-length *lyn* cDNA failed to reveal any gross alterations to the genomic structure of the *lyn* gene in J2E-NR cells (data not shown). However, when mRNA levels were examined by Northern

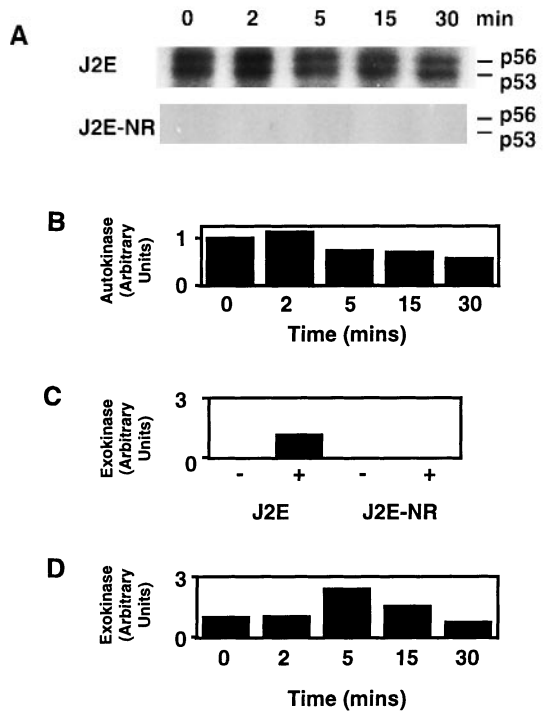

Fig. 2. J2E-NR cells have less lyn kinase activity than J2E cells. (**A**) Lysates (1.5 mg) were harvested from J2E and J2E-NR cells stimulated with epo for 0–30 min, immunoprecipitated with goat anti-lyn antibodies and subjected to an autokinase reaction before separation by electrophoresis and autoradiography. (**B**) Quantitation of J2E autokinase assay shown in (A). (**C**) Lysates (1.5 mg) from unstimulated J2E and J2E-NR cells were immunoprecipitated with rabbit anti-lyn antibodies and subjected to an exokinase reaction in the presence (+) or absence (–) of a lyn peptide substrate. (**D**) Lysates (1.5 mg) were harvested from J2E cells stimulated with epo for 0–30 min, immunoprecipitated with goat anti-lyn antibodies and subjected to an exokinase reaction in the presence of a lyn peptide substrate before separation by electrophoresis. Incorporation of [γ-^{32}P]ATP into the peptide was quantitated by densitometry and expressed as arbitrary units of exokinase activity relative to the amount of lyn protein.

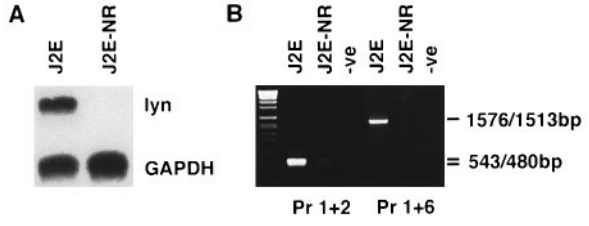

Fig. 3. Lyn mRNA is reduced in J2E-NR cells. (**A**) Northern blot analysis of poly(A)$^+$ RNA isolated from J2E and J2E-NR cells. Poly(A)$^+$ RNA (2 μg) was hybridized sequentially with ^{32}P-labelled *lyn* and GAPDH probes. (**B**) RT–PCR analysis of J2E and J2E-NR mRNA using specific lyn PCR primers 1+2 and 1+6. '–ve' represents the PCR negative control (no DNA). Molecular weight markers are shown in the left lane. The position and expected size of the *lyn* cDNA products are indicated.

blotting of poly (A)$^+$ RNA, no transcripts were detected in J2E-NR cells, even after prolonged exposure (Figure 3A). In contrast, *lyn* mRNA could readily be detected in the J2E cells. In an attempt to detect the presence of any *lyn* transcripts in J2E-NR cells, the more sensitive technique of reverse transcriptase–polymerase chain reaction (RT–PCR) was employed. Figure 3B shows that some *lyn* mRNA could be found in J2E-NR cells and that the

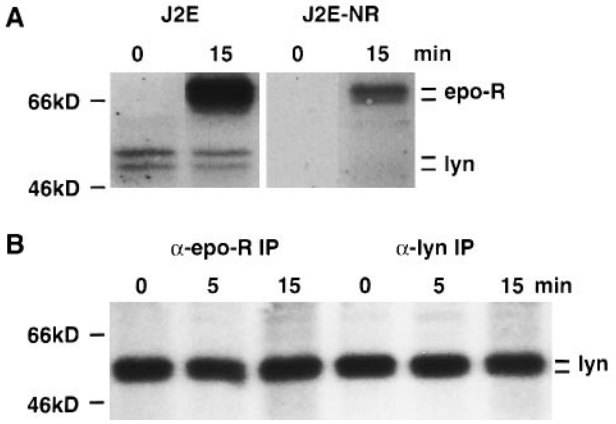

Fig. 4. Lyn associates with the epo-R in J2E cells. (**A**) Lysates (3 mg) from J2E and J2E-NR cells stimulated with epo (5 U/ml) for 0–15 min were immunoprecipitated with anti-epo-R antibodies and immunoblotted with anti-phosphotyrosine antibodies. The position of the epo-R and lyn proteins are indicated, as are the molecular weight markers. (**B**) Lysates (1.5 mg) from J2E cells stimulated with epo (5 U/ml) for 0–15 min were immunoprecipitated (IP) with anti-epo-R or rabbit anti-lyn antibodies and subjected to an autokinase reaction. The positions of lyn proteins are indicated, as are the molecular weight markers.

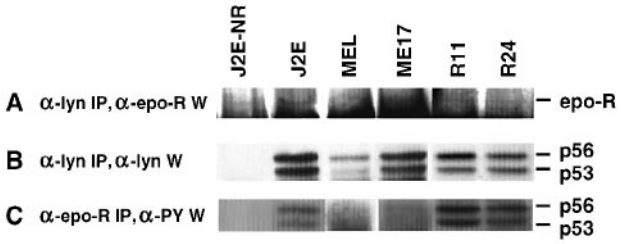

Fig. 5. Lyn associates with the epo-R in erythroid cell lines. Lysates (3 mg) from J2E-NR, J2E, MEL, ME17, R11 and R24 cells were immunoprecipitated (IP) with goat anti-lyn antibodies and immunoblotted (W) with anti-epo-R (**A**) or rabbit anti-lyn antibodies (**B**). Note that the anti-epo-R antibody does not immunoblot particularly well. (**C**) Lysates were also immunoprecipitated with anti-epo-R antibodies and immunoblotted with anti-phosphotyrosine antibodies.

fragments were of the expected size. This result shows that although transcripts emanating from the *lyn* gene were drastically diminished, the size of the mRNA was not noticeably altered. These data are consistent with the marked reduction in normal lyn protein in J2E-NR cells (Figure 1).

Lyn is part of the epo-R complex

We have previously shown that two tyrosine-phosphorylated proteins co-immunoprecipitated with the epo-R in the J2E cells (Tilbrook *et al.*, 1996a). These proteins migrated precisely to the position of the lyn protein doublet in J2E cells, and were absent from J2E-NR cells (Figure 4A). Interestingly, these proteins were associated with the epo-R in J2E cells before, and after, epo stimulation. In addition, the epo-R-associated proteins possessed autokinase activity and migrated to the same position as p53 and p56 lyn (Figure 4B). The interaction between the epo-R and lyn could also be shown in ME17, R11 and R24 erythroid cell lines (Figure 5). The presence of the epo-R was demonstrated in anti-lyn (goat) immunoprecipitations (Figure 5A) while, conversely, the tyrosine-

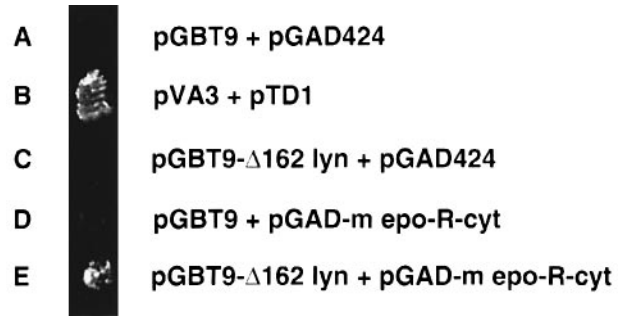

Fig. 6. Yeast two-hybrid analysis demonstrating an interaction between lyn and epo-R. *Saccharomyces cerevisiae* CG-1945 clones were assessed for their ability to grow on HIS^- plates following transformation with (**A**) pGBT9 and pGAD424, vector negative control; (**B**) pTD1 and pVA3, positive interaction control; (**C**) pGAD424 and pGBT9-Δ162 lyn, Δ162 lyn interaction control; (**D**) pGBT9 and pGAD-m epo-R-cyt, epo-R interaction control; (**E**) pGBT9-Δ162 lyn and pGAD-m epo-R-cyt; Δ162 lyn and epo-R test interaction.

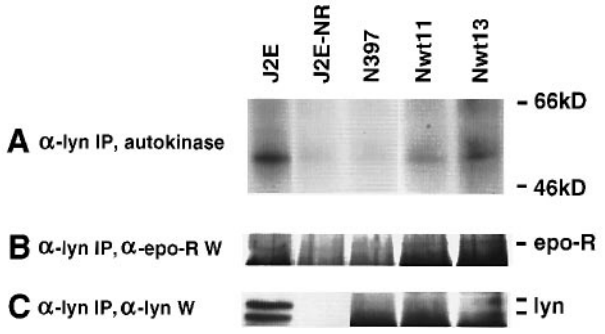

Fig. 7. Lyn kinase activity in J2E-NR cells infected with wild-type and dominant negative lyn. (**A**) Lysates (2 mg) from J2E, J2E-NR, N397, Nwt11 and Nwt13 cell lines were immunoprecipitated (IP) with goat anti-lyn antibodies and subjected to an autokinase reaction. Lysates (3 mg) from J2E, J2E-NR, N397, Nwt11 and Nwt13 cell lines were immunoprecipitated with goat anti-lyn antibodies and immunoblotted (W) with anti-epo-R (**B**) or rabbit anti-lyn antibodies (**C**). Representative examples of six N397 and 12 Nwt clones are shown.

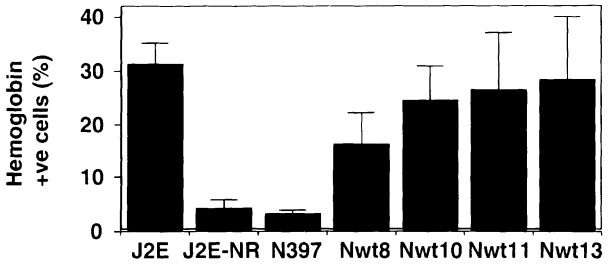

Fig. 8. J2E-NR cells expressing wild-type lyn synthesize haemoglobin in response to epo. Cell lines were stimulated with epo for 48 h and haemoglobin synthesis measured by DAF staining. The results shown are the mean ± SD (n = 3) of DAF stained cells from J2E, J2E-NR, one of six N397 and four of 12 different Nwt cell lines.

phosphorylated p53/p56 doublet was present in anti-epo-R immunoprecipitations (Figure 5C). These data suggest that the src-like kinase lyn forms part of the epo-R complex in erythroid cells. Direct detection of lyn in an anti-epo-R immunoprecipitation was not possible due to cross-reactivity of the immunoglobulin bands of rabbit anti-lyn and rabbit anti-epo-R antibodies which co-migrate with lyn, and the inability of the goat anti-lyn antibody to immunoblot. An interaction between lyn and epo-R was not seen in J2E-NR or MEL cells. The absence in the MEL cells is probably due to the inability of the truncated epo-R in the MEL cell line (Bittorf *et al.*, 1996) to interact with lyn.

The yeast two-hybrid system was utilized to further analyse the association of lyn with the epo-R complex, and to determine if this interaction was direct and not via a linker molecule. Initial experiments with the full-length lyn protein and the cytoplasmic domain of the epo-R failed to reveal conclusively any interactions between the two proteins. This is not uncommon in yeast two-hybrid analyses as 'steric hindrance' often occurs with large fusion molecules (Fields and Sternglanz, 1994). However, when the first 162 amino acids of lyn, containing only the unique and SH3 binding domains (pGBT9-Δ162 lyn), were assayed in conjunction with the cytoplasmic domain of the epo-R (pGAD-m epo-R-cyt), an interaction between these two molecules could be demonstrated. Figure 6 shows the association of these two proteins by the ability of yeast containing these constructs to grow on plates lacking histidine (HIS^-). Thus, the biochemical and yeast two-hybrid system data indicate that lyn is associated with the epo-R in J2E cells. The absence of active lyn kinase in J2E-NR cells supports our observation that lyn is a key signalling molecule linked to the epo-R as it correlates closely with the inability of J2E-NR cells to terminally differentiate in response to epo.

Expression of lyn in J2E-NR cells restores the ability to differentiate

Since the J2E-NR cells were severely deficient in lyn, we speculated that this was responsible for their inability to differentiate. To test this hypothesis, wild-type lyn (pRN-wt lyn) and a dominant negative form of lyn (pRN-Y397F lyn) were expressed in J2E-NR cells using a retroviral vector and amphotropic infection. The dominant negative lyn was created by a tyrosine to phenylalanine substitution at residue 397. Resultant lines were designated Nwt or N397 for wild-type and dominant negative lyn respectively, and six independent clones were analysed. Although expression of lyn protein in the J2E-NR cells was increased following infection with both retroviral constructs, it was still less than that of the parental J2E cells (Figure 7). Nevertheless, autokinase activity and lyn interacting with the epo-R could now be detected in J2E-NR cells expressing wild-type lyn but not in clones expressing the dominant negative lyn (Figure 7).

J2E-NR cells were originally identified by their inability to synthesize haemoglobin, as demonstrated by benzidine staining (Klinken and Nicola, 1990). In addition, no haemoglobin could be detected by spectral analyses (data not shown). To determine if the introduction of lyn had corrected the defect in the J2E-NR line, cells were exposed to epo and monitored for haemoglobin synthesis. Significantly, Nwt cells now responded to epo by manufacturing the oxygen carrier (Figure 8). However, levels of haemoglobin synthesized by Nwt cells were lower than J2E cells, and required the more sensitive diaminofluorene (DAF) stain for detection (Callus *et al.*, 1995). Presumably, better expression of exogenous lyn would lead to greater haemoglobin production. Spectral scans revealed that only haemoglobin (with absorbance peaks of 560 and 430 nm)

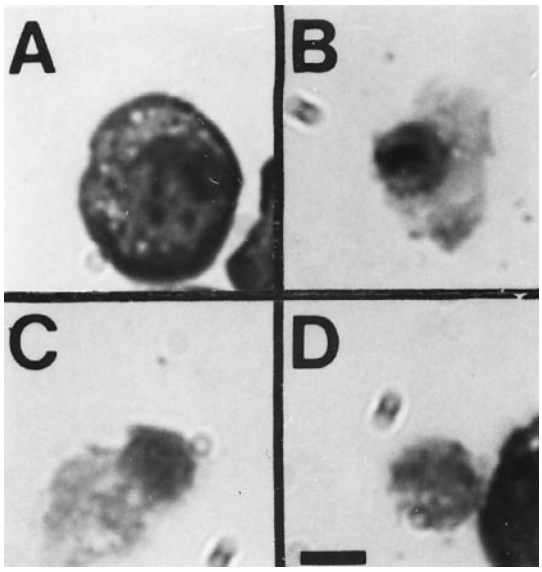

Fig. 9. Morphological maturation of J2E-NR cells expressing wild-type lyn. J2E-NR or Nwt cells were stimulated with epo for 72 h before cytocentrifugation onto glass slides and immersion in Wright's stain. Only proerythroblasts were observed in J2E-NR cultures (**A**); however some polychromatic, orthochromatic, enucleating erythrocytes (**B** and **C**) and reticulocytes (**D**) were observed in all six Nwt cell lines tested. The bar represents 10 μm.

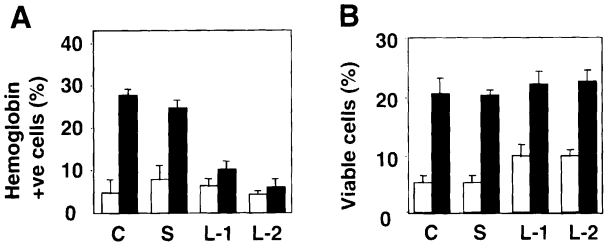

Fig. 10. Antisense lyn oligonucleotides reduce the ability of J2E cells to differentiate. J2E cells were treated with antisense lyn (L-1 or L-2) or control sequence (S) oligonucleotides for 72 h before epo stimulation. Cells not exposed to oligonucleotides were used as a control (C). The effects on differentiation and viability were examined 48 h after epo stimulation. Untreated cells were represented by open columns; epo-stimulated cells are represented by solid columns. Differentiation was measured by benzidine staining (**A**) and viability by eosin exclusion (**B**). Each value represents the mean ± SD (n = 3).

was present in the Nwt cells, unlike the J2E cells which contain primarily oxyhaemoglobin, with absorbance peaks of 577, 540 and 413 nm (Busfield *et al.*, 1995; data not shown).

In addition to synthesizing haemoglobin in response to epo, J2E cells are able to differentiate morphologically from proerythroblasts through to reticulocytes (Klinken *et al.*, 1988; Busfield and Klinken, 1992; Busfield *et al.*, 1993a,b,c). In contrast, the J2E-NR cells do not mature morphologically in response to epo (Klinken and Nicola, 1990). However, when wild-type lyn was reintroduced, the Nwt cells demonstrated signs of morphological differentiation after exposure to epo. Polychromatic and orthochromatic erythroblasts with condensed nuclei and reduced cytoplasmic volume were evident, as were enucleating cells and reticulocytes (Figure 9). While <5% of these cells underwent morphological maturation compared with 30–40% by J2E cells (Busfield *et al.*, 1993b), these results showed that J2E-NR cells expressing wild-type lyn were able to progress through each stage of erythroid terminal differentiation.

Reduction in lyn kinase activity impairs differentiation of J2E cells

As we had demonstrated that introduction of lyn into J2E-NR cells corrected the differentiation defect, the role of lyn in epo-induced maturation of the parental cell line was examined. Two strategies were employed to suppress lyn activity in J2E cells: the first involved the use of antisense oligonucleotides and the second utilized the introduction of a dominant negative lyn. Antisense oligonucleotides, L-1 and L-2, were designed to the 5′ end (immediately adjacent to the start codon) and the 3′ untranslated region of lyn respectively. Cells grown in the presence of these oligonucleotides for 72 h showed a 65–70% reduction in lyn protein (data not shown). This reduction is comparable with the 50% decrease reported by Scheuermann *et al.* (1994) with the L-1 oligonucleotide in B lymphoma cells. In contrast, treatment with the control sequence oligonucleotide (S) did not reduce lyn protein levels (data not shown). Importantly, normal cell growth was not affected by treatment with any of the oligonucleotides. However, when the cells were exposed to epo, differentiation measured by benzidine staining of haemoglobin was noticeably restricted with both antisense lyn oligonucleotides, but not with the control sequence oligonucleotide (Figure 10A). In contrast, neither of the antisense oligonucleotides inhibited the ability of epo to support viability in J2E cells (Figure 10B). The suppression of epo-induced differentiation by the L-1 oligonucleotide could also be demonstrated in the R24 cell line. Epo stimulated haemoglobin production in R24 cells from 7% to 17% (see Figure 12D); although treatment with L1 had no impact on background haemoglobinization (8%), it did restrict epo-stimulated haemoglobin synthesis to 9%.

The second approach used to reduce the amount of active lyn in the J2E cells was to express a dominant negative form of lyn in the cells. Using amphotropic retroviral infection, the dominant negative lyn construct described earlier (pRN-Y397F lyn) was introduced into J2E cells and the resulting cell lines designated J397. As a control, wild-type lyn (pRN-wt lyn) was also introduced into J2E cells, generating Jwt lines. Six independent clones containing each construct were isolated. The activity of lyn and its interaction with the epo-R was examined in each of the infected cell lines. Figure 11 shows that J397 cell lines had markedly reduced lyn autokinase activity; tyrosine-phosphorylated lyn was virtually undetectable and very low levels of tyrosine-phosphorylated lyn were associated with the epo-R. In contrast, Jwt cell lines had slightly higher lyn autokinase activity than control J2E cells, they contained more tyrosine-phosphorylated lyn, and had increased amounts of tyrosine-phosphorylated lyn in the epo-R complex. These data support our approach of using a dominant negative lyn to reduce lyn kinase activity in J2E cells.

When these cell lines were exposed to epo, the J397 cell lines were severely restricted in their capacity to produce haemoglobin, while the Jwt cell lines synthesized slightly more haemoglobin than the parental J2E cells

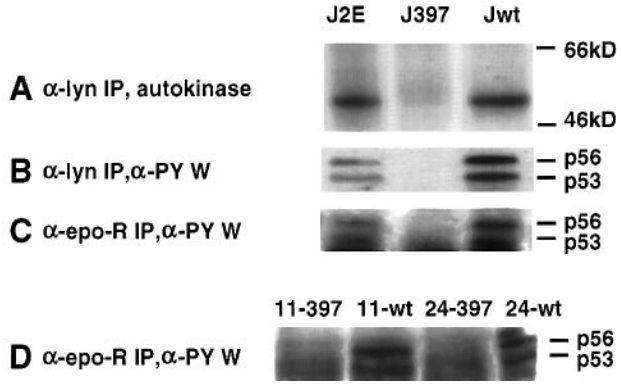

Fig. 11. Lyn kinase activity in transfected erythroid cell lines. (**A**) Lysates (2 mg) from cell lines J2E, J397 and Jwt were immunoprecipitated (IP) with goat anti-lyn antibodies and subjected to an autokinase reaction. (**B**) Lysates (2 mg) from cell lines J2E, J397 and Jwt were immunoprecipitated with rabbit anti-lyn antibodies and immunoblotted (W) with anti-phosphotyrosine antibodies. (**C**) Lysates (2 mg) were immunoprecipitated with anti-epo-R antibodies and immunoblotted with anti-phosphotyrosine antibodies. (**D**) Lysates (3 mg) from derivatives of cell lines R11 (11-397 and 11-wt) and R24 (24-397 and 24-wt) were immunoprecipitated with anti-epo-R antibodies and immunoblotted with anti-phosphotyrosine antibodies. A representative of six 397 and wt lyn clones is shown. The positions of p53 and p56 lyn and molecular size markers are indicated.

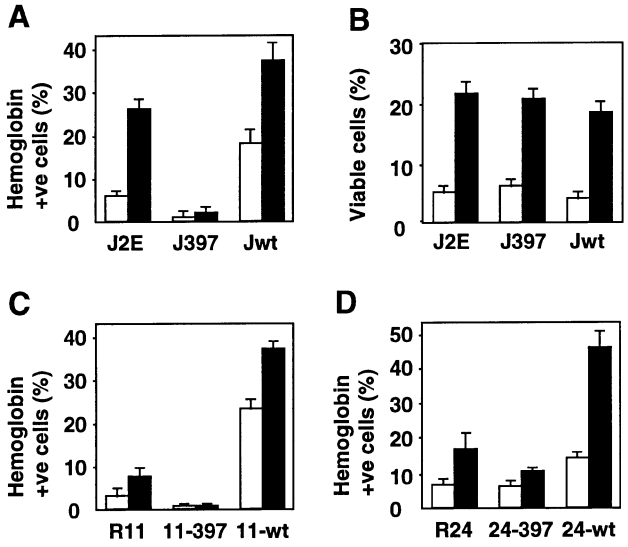

Fig. 12. Dominant negative lyn reduces the ability of J2E, R11 and R24 cells to differentiate. The effect of epo on differentiation (A, C and D) and viability (B) in J2E, J397 and Jwt (**A** and **B**), R11, 11-397, 11-wt (**C**) and R24, 24-397, 24-wt (**D**) cell lines. A representative of six clones is shown. Cells were grown in the presence (solid columns) or absence (open columns) of epo (5 U/ml). Differentiation of the cells was measured by benzidine staining (A, C and D) and viability by eosin exclusion (B). Each value represents the mean ± SD (n = 3).

(Figure 12A). This result demonstrates that the reduction in active lyn kinase in the J397 cell lines inhibited epo-induced differentiation and corroborates the observations with antisense oligonucleotides (Figure 10A and B). Interestingly, haemoglobin production by unstimulated J397 was also reduced while, conversely, benzidine staining of uninduced Jwt cell lines was elevated compared with J2E cells (Figure 12A). In contrast with the differentiation response, the ability of epo to maintain viability was not affected by the presence of either lyn construct (Figure 12B).

The importance of lyn to differentiation was tested in two other epo-responsive cell lines (R11 and R24). Both dominant negative (pRN-Y397F) lyn and wild-type (pRN-wt) lyn were introduced into these cells. R11 and R24 did not differentiate appreciably with epo (Figure 12C and D). Nevertheless, Figure 12C and D shows that the Y397F lyn suppressed haemoglobin production in both lines (11-397 and 24-397), while enhanced differentiation was noted with exogenous wild-type lyn (11-wt and 24-wt). The reduction in differentiation with dominant negative lyn coincided with decreased association between tyrosine-phosphorylated lyn and epo-R complex (Figure 11D).

Taken together, these experiments demonstrate that a reduction in active lyn protein results in a reduced capacity of J2E, R11 and R24 cells to differentiate in response to epo; however, viability of J2E cells in the presence of epo is unaffected. These data support our hypothesis that lyn is an important component of the epo-R signal transduction complex in erythroid cells.

Discussion

Epo binding to its cognate receptor initiates a series of intracellular signalling events which result in the maturation, proliferation and prevention of apoptosis in erythroid cells (Krantz, 1991; Jelkman, 1992; Koury and Bondurant, 1992). J2E erythroid cells undergo enhanced proliferation, differentiate and remain viable in response to epo while the hormone only supports the viability of J2E-NR cells, a mutant J2E clone (Busfield and Klinken, 1992; Tilbrook *et al.*, 1996a). Therefore, J2E and J2E-NR cells provide a useful model system to partially dissect the epo signalling pathways for terminal differentiation from the maintenance of viability. Here, we report that the defective signalling molecule in J2E-NR cells is the tyrosine kinase lyn. Moreover, we demonstrate for the first time the key role of lyn in epo signalling for maturation, but not for cell survival.

Lyn mRNA and protein content were dramatically reduced in J2E-NR cells. Strikingly, the ability of J2E-NR cells to differentiate in response to epo was restored when lyn was re-expressed in these cells. Not only were the cells able to manufacture haemoglobin, but they were also able morphologically to differentiate in response to epo. These results demonstrate that lyn is responsible for the defect in J2E-NR cells which prevents epo-induced differentiation. It is significant that lyn has been implicated in the phosphorylation of PI-3K, GAP and MAP kinases (Cichowki *et al.*, 1992; Yamanashi *et al.*, 1992; Corey *et al.*, 1993; Pleiman *et al.*, 1993) as none of these signal transducers is phosphorylated in J2E-NR cells following epo stimulation (Tilbrook *et al.*, 1996a).

The importance of lyn in erythroid differentiation was confirmed by introducing antisense lyn oligonucleotides into J2E and R24 cells, and a dominant negative lyn into J2E, R11 and R24 cells. The levels of active lyn protein were reduced by both experimental approaches and the cells were restricted in their ability to differentiate with epo stimulation. Although the reduction in lyn activity suppressed terminal differentiation of J2E cells, it did not hinder the ability of epo to maintain viability in the absence of serum. This result is consistent with the

observation that J2E-NR cells remain viable in the presence of epo (Tilbrook *et al.*, 1996a), despite their lack of lyn expression. Interestingly, Scheuermann *et al.* (1994) demonstrated that lyn is required for the control of cell division in B-cells, but not for the signal leading to apoptosis. There is mounting evidence in other systems which supports the contention that signalling for viability can be separated from proliferation and differentiation pathways (Okuda *et al.*, 1994; Boise *et al.*, 1995; Inhorn *et al.*, 1995; Kinoshita *et al.*, 1995). We have demonstrated recently that MAP kinase, like lyn, is not essential for epo-induced viability but is required for maturation (Tilbrook *et al.*, 1996a; Chappell *et al.*, 1997). In combination, these data indicate that distinct signalling pathways exist for epo-enhanced viability and differentiation with lyn and MAP kinase participating in the cascade leading to cellular maturation.

The severe down-regulation of lyn in J2E-NR cells may be due to a promoter/enhancer defect in the gene. There were no gross alterations in the genomic structure of *lyn* and both the mRNA and protein were of the anticipated size (Stanley *et al.*, 1991; Yamanashi *et al.*, 1991b; Yi *et al.*, 1991). We speculate, therefore, that the fault may lie with the capacity to transcribe the gene—either through a subtle mutation in the promoter/enhancer region or through the loss of an essential transcription factor. However, it has also been suggested that the J2E-NR cells may have 'de-differentiated' to a less mature form than J2E cells since they no longer express the surface marker Ter 119 (Keil *et al.*, 1995). An alternative explanation is that *lyn* is not switched on in immature erythroid cells. Consequently, early erythroid precursors may be kept alive by epo but do not differentiate until *lyn* has been activated.

Witthuhn *et al.* (1993) first demonstrated an association between JAK2 and the epo-R. Their study, conducted with myeloid DA-3 cells transfected with the epo-R (which do not undergo erythroid differentiation), did not show an interaction between lyn and the epo-R. We have good evidence to suggest an interaction exists between the epo-R and lyn in erythroid cells. First, epo-R protein was detected in anti-lyn immunoprecipitations, a tyrosine-phosphorylated protein doublet co-immunoprecipitated with the epo-R, the proteins possessed autokinase activity and migrated to exactly the same position as lyn. These proteins were absent in J2E-NR cells. Second, the yeast two-hybrid system showed an interaction between the cytoplasmic domain of the epo-R and the amino-terminal portion of lyn containing the unique domain and SH3 domain. Additionally, when dominant negative lyn was introduced into J2E cells and two other erythroid cell lines (R11 and R24), much less active lyn was associated with the epo-R, which correlated with their reduced ability to differentiate in response to epo. These results suggest a crucial role for lyn in the epo-R complex, subsequent signalling and erythroid maturation. Whether the role for lyn in erythropoietin signalling shown here in transformed cell lines can be extended to normal erythroid precursors requires further experimentation.

A recent study on *lyn*–/– mice demonstrated that lyn is indispensable for B-cell receptor and FcεRI signalling (Hibbs *et al.*, 1995b). *Lyn*-deficient mice succumb at an early age as a result of autoimmune disease. Although *lyn*-deficient mice die with anaemia, this is believed to be a secondary defect caused by the autoimmune disease. The mice develop severe glomerulonephritis and are likely to have impaired epo production as a consequence of kidney degeneration. As *lyn*-deficient mice have normal levels of erythrocytes, it appears that *lyn* may be dispensable for erythroid maturation. However, it is possible that another member of the *src* family can functionally substitute for the absence of *lyn*. Functional redundancy within this family has been suggested by the milder than expected phenotype associated with mice deficient in *src* (Soriano *et al.*, 1991), *fyn* (Appleby *et al.*, 1992; Stein *et al.*, 1992), *yes* (Grant *et al.*, 1992) and *hck* and *fgr* (Lowell *et al.*, 1994). *In vitro* studies with a *lyn*$^-$ DT40 B-cell line, have shown that lyn function can be substituted by fyn or lck, but not src (Takata *et al.*, 1994). In this light it will be interesting to determine if the kinase activity of other src family members is elevated in lyn-deficient erythroid cells.

An interesting parallel can be drawn between the lack of lyn in J2E-NR cells preventing differentiation, and normal erythrocyte production in *lyn*–/– mice. An MEL cell line which fails to express transcription factor p45 NF-E2 does not synthesize β-globin (Lu *et al.*, 1994); however, enforced expression of NF-E2 partially restores the capacity to produce globins. Conversely, reduction of NF-E2 in MEL cells inhibits globin expression (Kotkow and Orkin, 1995). In marked contrast with these *in vitro* studies, targeted disruption of the p45 NF-E2 gene has little effect on erythrocyte production and haemoglobin synthesis in knockout mice (Shivdasani and Orkin, 1995). As the NF-E2 complex recognizes an extended AP-1 motif (Andrews *et al.*, 1993), it has been postulated that functional redundancy may compensate for the loss of NF-E2 (Shivdasani and Orkin, 1995). Thus, loss of NF-E2 or lyn in erythroid cell lines prevents terminal differentiation, whereas the absence of these genes in mice does not appreciably impede the production of red blood cells. These observations may provide vital clues as to how cells can overcome functional defects early in development, but are unable to compensate for these faults as they become more committed and enter the final stages of differentiation.

Materials and methods

Cell culture

J2E (Klinken *et al.*, 1988), J2E-NR (Klinken and Nicola, 1990), the MEL cell line F4N (Dube *et al.*, 1975), ME17 (Cory *et al.*, 1991), R24 and R11 (Metz *et al.*, 1995) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 7% fetal calf serum. Cell viability was determined by eosin exclusion (Busfield and Klinken, 1992) and haemoglobin synthesis determined by benzidine or DAF staining (Cooper *et al.*, 1974; Callus *et al.*, 1995). Cell morphology was examined by cytocentrifuging cells onto glass slides and immersion in Wright's stain. For haemoglobin spectra, cells (5×10^7) were lysed in water for 1 h on ice and spectral scans performed between 350 and 700 nm.

Cell labelling, immunoblotting, immunoprecipitation and kinase assays

Cells were labelled with [^{35}S]methionine/cysteine as described previously (Tilbrook *et al.*, 1996b). Cells were serum-deprived, stimulated with epo and lysed in buffer (150 mM NaCl, 1% Triton X-100, 20 mM Tris, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) as described previously (Tilbrook *et al.*, 1996a). Immunoprecipitations were conducted with 1–5 mg protein with anti-epo-R (187; Yoshimura *et al.*, 1990) or anti-lyn [L40 (Boulet *et al.*, 1992) or sc15Goat (Santa Cruz

Biotechnology Inc., CA, USA)] antibodies and protein A–Sepharose beads. Immunoprecipitated proteins or cell lysates (100 μg) were separated by SDS–PAGE, transferred to nitrocellulose membranes and analysed by immunoblotting with anti-lyn [L40 (Boulet *et al.*, 1992) or sc15 (Santa Cruz Biotechnology Inc., CA, USA)] or anti-phosphotyrosine [4G10 (Upstate Biotechnology, NY, USA)] antibodies, followed by horseradish peroxidase-conjugated antibodies. Visualization was by enhanced chemiluminescence (Amersham, Bucks, UK).

For kinase reactions, cells were lysed (20 mM Tris, pH 7.5, 0.1% Nonidet P-40, 10 mM β-glycerophosphate, 10 mM NaF, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin); 1.5 mg protein was then immunoprecipitated with anti-lyn (sc15Goat) antibodies and protein A–Sepharose beads. Immunoprecipitates were washed three times in lysis buffer, then twice in kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM EGTA, 1 mM Na_3VO_4). For autokinase reactions, kinase buffer (25 μl) and 1 μl [γ-^{32}P]ATP were added to the immunoprecipitates and incubated at 30°C for 20 min. Phosphorylated proteins were then separated by SDS–PAGE, transferred to a nitrocellulose membrane and visualized on a Molecular Dynamics PhosphorImager. For exokinase reactions, 2 μl substrate peptide [sc 3021 (Santa Cruz Biotechnology, CA, USA)] was added to the reaction before separation on a gradient SDS–PAGE gel, drying and autoradiography.

Antisense lyn oligonucleotide treatment

Phosphorothioate-modified oligonucleotides were synthesized by Macromolecular Resources (Fort Williams, CO, USA). The two lyn antisense oligonucleotides were L-1: 5′-CAT ATT TCT CGC TCG TGG TG-3′ (Scheuermann *et al.*, 1994), which complements nucleotides 219–238 (Stanley *et al.*, 1991) and L-2: 5′-CCA TTG AGC GGC CAC CC-3′, which complements nucleotides 1922–1942 (Stanley *et al.*, 1991). The control oligonucleotide sequence (S) was: 5′-CGC GCG CTC GCG CAC CC-3′. J2E cells were treated with 10 μM oligonucleotide for 72 h before addition of epo. During this time the cells were diluted to 3×10^4 cells/ml and the oligonucleotide replenished daily as described previously (Tilbrook *et al.*, 1996b). For differentiation studies, cells at 3×10^4/ml were stimulated with 5 U/ml epo for 48 h in the presence of 10 μM oligonucleotide. For viability studies, cells were washed in DMEM, resuspended at 3×10^5 cells/ml with 10 μM oligonucleotide and stimulated with 5 U/ml epo for 48 h.

Amphotropic viral infection of J2E and J2E-NR cells with retroviral lyn constructs

Site-directed mutagenesis (Kunkel, 1985) was used to introduce unique *Bam*HI and *Bgl*II sites respectively in the 5′ and 3′ untranslated regions of the mouse *lyn* cDNA (Stanley *et al.*, 1991) for subcloning. The modified full-length *lyn* cDNA was subcloned into the *Bam*HI site of the expression vector pCDNA1Amp (pCALyn). A dominant negative form of lyn was generated by mutating the autophosphorylated tyrosine at amino acid 397 to phenylalanine (pCALynY397F). The retroviral vector pRuftkNeo (Rayner and Gonda, 1994) was used to express wild-type (pRN-wt lyn) and dominant negative lyn (pRN-Y397F lyn). These constructs were generated by subcloning the 1.56 kb *Bam*HI–*Pst*I fragments from pCALyn and pCALynY397F into *Bam*HI–*Hin*dIII sites of pRufNeo with a 40 bp *Pst*I–*Hin*dIII adaptor. The packaging cell line PA317 was then transfected with the constructs by calcium phosphate precipitation (Chen and Okayama, 1987) and supernatants containing amphotropic retroviruses were used to infect J2E, J2E-NR, R11 and R24 cells. Cells were selected in G418 (Sigma, St Louis, MO, USA) before subcloning in methyl cellulose (Klinken *et al.*, 1988). Between 6 and 12 independent colonies were isolated for each construct and integration of the cDNA construct was confirmed by PCR analysis using lyn primers 4 (5′-TCC CAA ACC TCA GAA GCC AT-3′) and 5 (5′-GGC TCC TCC TTG GTG ACC AC-3′) which span the exon 8/exon 9 boundary of the *lyn* gene (Hibbs *et al.*, 1995a) and whose 5′ ends are positioned at nucleotides 919 and 1176 respectively in the *lyn* cDNA (Stanley *et al.*, 1991).

Northern blotting and PCR analysis

Total cytoplasmic RNA was isolated from J2E and J2E-NR cells by the method of Chomczynski and Sacchi (1987). Poly(A)$^+$ RNA, purified using the PolyATract isolation system (Promega, WI, USA), was hybridized with a ^{32}P-labelled 284 bp fragment derived from the unique domain of *lyn* (Stanley *et al.*, 1991). *Lyn* cDNA was amplified by RT–PCR as described previously (Bittorf *et al.*, 1996) using primers 1 (5′-CAC CAC GAG CGA GAA ATA TG-3′), 2 (5′-CAC CAT GCA TAG GGT CAT AA-3′) and 6 (5′-AAT AGG CTA GTC TCC ATG CC-3′) whose 5′ ends are positioned at nucleotides 221, 767 and 1795 respectively in the *lyn* cDNA (Stanley *et al.*, 1991).

Yeast two-hybrid analysis

A 1.56 kb *Bam*HI–*Pst*I fragment from pCALyn encoding full-length *lyn* and a 0.5 kb *Hin*dIII fragment encoding the amino-terminal 162 amino acids of lyn were subcloned in-frame into the GAL4 DNA binding domain vector pGBT9 (Clonetech, Palo Alto, CA, USA). The resultant plasmids were designated pGBT9-lyn and pGBT9-Δ162 lyn respectively. A 0.8 kb *Bgl*II–*Cla*I fragment encoding the cytoplasmic domain of the murine epo-R (amino acids 279–507) was subcloned in-frame into the GAL4 activation domain vector pGAD424 (Clonetech, Palo Alto, CA, USA) and called pGAD-m epo-R-cyt. The handling, transformation and analysis of the *Saccharomyces cerevisiae* strain CG-1945 was performed according to the manufacturer's instructions (Matchmaker Two-Hybrid System Protocol; Clonetech, Palo Alto, CA, USA). In brief, cells were co-transformed with the following pairs of plasmids; pGBT9 and pGAD424 (vector negative control), pTD1 and pVA3 (positive interaction control between p53 and the SV40 large T antigen), pGAD424 and pGBT9-Δ162 lyn (Δ162 lyn interaction control), pGBT9 and pGAD-m epo-R-cyt (epo-R interaction control), and pGBT9-Δ162 lyn and pGAD-m epo-R-cyt (Δ162 lyn and epo-R test interaction). The transformants were plated onto synthetic dextrose agar plates lacking L-leucine and L-tryptophan. The resultant colonies were then re-plated onto similar plates lacking L-histidine (HIS$^-$). After 3–4 days at 30°C, colony growth was assayed visually.

Acknowledgements

Recombinant human epo (Eprex) was a generous gift from Dr J.Adams (Jansen-Cilag), and anti-epo-R antibodies and epo-R cDNA were kindly donated by Dr S.Watowich (M.D. Anderson Cancer Center, Houston, TX, USA). The authors also thank Drs S.Cory, J.Adams and T.Metz for kindly providing cell lines ME17, R11 and R24, and Dr T.Gonda for the Ruftkneo vector. This work was supported by grants from NH&MRC (#96-0571), AMRAD, Raine Medical Research Foundation and the Australian Kidney Foundation. M.L.H. is supported by a Queen Elizabeth II fellowship from the Australian Research Council.

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Received on July 2, 1996; revised on November 11, 1996