The glucocorticoid receptor is a key regulator of the decision between self-renewal and differentiation in erythroid progenitors

Oliver Wessely, Eva-Maria Deiner, Hartmut Beug¹ and Marieke von Lindern²

Institute of Molecular Pathology, Dr Bohr Gasse 7, A-1030 Vienna, Austria

²Present address: Institute of Hematology, Erasmus University, Box 1738, NL-3000DR, Rotterdam, The Netherlands

¹Corresponding author

During development and in regenerating tissues such as the bone marrow, progenitor cells constantly need to make decisions between proliferation and differentiation. We have used a model system, normal erythroid progenitors of the chicken, to determine the molecular players involved in making this decision. The molecules identified comprised receptor tyrosine kinases (c-Kit and c-ErbB) and members of the nuclear hormone receptor superfamily (thyroid hormone receptor and estrogen receptor). Here we identify the glucocorticoid receptor (GR) as a key regulator of erythroid progenitor self-renewal (i.e. continuous proliferation in the absence of differentiation). In media lacking a GR ligand or containing a GR antagonist, erythroid progenitors failed to self-renew, even if c-Kit, c-ErbB and the estrogen receptor were activated simultaneously. To induce self-renewal, the GR required the continuous presence of an activated receptor tyrosine kinase and had to cooperate with the estrogen receptor for full activity. Mutant analysis showed that DNA binding and a functional AF-2 transactivation domain are required for proliferation stimulation and differentiation arrest. *c-myb* was identified as a potential target gene of the GR in erythroblasts. It could be demonstrated that Δc -Myb, an activated c-Myb protein, can functionally replace the GR

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Introduction

Lipophilic hormones including steroids have important functions in regulating development, cell differentiation and homeostasis. They bind and activate intracellular receptors, which are direct modulators of transcription (for recent reviews, see Beato *et al.*, 1995; Mangelsdorff *et al.*, 1995). One of the best studied nuclear hormone receptors is the glucocorticoid receptor (GR). The GR is expressed in a wide variety of cell types and is involved in the control of various physiological processes such as the regulation of carbohydrate, lipid and protein metabolism (for review, see Müller and Renkawitz, 1991) and the modulation of the immune system, for instance in inflammation (Boumpas *et al.*, 1993; Dewaal, 1994). The

generation of GR-negative mice showed that the GR is essential for the proper development of several organ systems such as the lung and the adrenal glands (Cole *et al.*, 1995).

Numerous mechanisms have been proposed by which the ligand-activated GR can modulate expression of target genes (reviewed by Beato *et al.*, 1995). Firstly, a GR homodimer can bind to glucocorticoid response elements (GREs) via their DNA binding (DBD) domain and enhance transcription via interaction of the activation function (AF)-2 domain with components of the basal transcription machinery, either directly (reviewed by Tsai and O'Malley, 1994) or via an emerging class of co-activators interacting with the AF-2 domain (see Cavaillès *et al.*, 1995, and references therein). Secondly, again via its AF-2 domain, the GR can activate transcription by rendering chromatin accessible to other transcription factors (see Espinàs *et al.*, 1995, and references therein). In yeast, SWI1, SWI2 and SWI3 are essential to allow transcriptional activation by the GR (Yoshinaga *et al.*, 1992). In animal cells, vertebrate homologs of the yeast *SWI2/SNF2/Drosophila brahma* genes exert a corresponding function (Muchardt and Yaniv, 1993; Chiba *et al.*, 1994). Finally, the GR can interact with other transcription factors like the AP-1 complex, GATA-1 and members of the NFκB family, such as Rel-A and p65 NFκB. These interactions result in mutual repression of transcriptional activation and occur independently of an active transactivation domain, as well as of DNA binding and dimerization (Chang *et al.*, 1993; Heck *et al.*, 1994; Ray and Prefontaine, 1994; Caldenhoven *et al.*, 1995).

Despite our detailed knowledge about the biochemistry of GR function, few systems exist in which regulation of a biologically relevant process by the GR can be studied at the molecular level. Here, we identify the GR as a key regulator of an important developmental decision in hematopoiesis. Hematopoietic progenitor cells generate large numbers of differentiated offspring (Sawada *et al.*, 1991). In addition, they sometimes generate and maintain a certain level of immature cells, which proliferate without apparent differentiation (in the following referred to as 'self-renewal'). To keep this balance, the cell has to regulate the decision between proliferation and differentiation closely. Such a mechanism not only occurs in pluripotent progenitor cells (Keller, 1992) but also in certain committed progenitors (Rolink and Melchers, 1991; Steinlein *et al.*, 1995), and its disturbance results in severe diseases such as anemias or leukemias (Sawyers *et al.*, 1991).

First insights into the potential molecules regulating proliferation and differentiation in hematopoietic progenitors arose from a disease model, lethal chick erythro-leukemias induced by the avian erythroblastosis virus (AEV). AEV encodes two cooperating oncoproteins,

v-ErbB and v-ErbA. v-ErbB is a mutated, constitutively activated version of the receptor tyrosine kinase c-ErbB, while v-ErbA is a mutated avian thyroid hormone receptor (TR) α (for recent reviews, see Hayman and Beug, 1992; Metz, 1994). Both v-ErbB and v-ErbA are thought to act by mimicking functions of normal, endogenous regulators belonging to the same gene families as the oncoproteins (Beug *et al.*, 1994). This has been demonstrated clearly for v- and c-ErbB. c-ErbB-expressing, normal erythroid progenitors continuously proliferate in the presence of ligand [mammalian transforming growth factor- α (TGF- α), Pain *et al.*, 1991; Hayman *et al.*, 1993]. These cells are indistinguishable from v-ErbB-transformed leukemic cells but differentiate terminally if the c-ErbB ligand is removed (Beug *et al.*, 1995).

Normal regulators with a biological function similar to that of v-ErbA were harder to identify. The endogenous c-ErbA/TR α is expressed in erythroid progenitors, but fails to induce proliferation and arrest differentiation, either with or without ligand (Schroeder *et al.*, 1992). Another member of the nuclear hormone receptor family, the estrogen receptor (ER), has been shown to cooperate with c-ErbB in the induction of self-renewal (Schroeder *et al.*, 1993). Recently, however, we showed that the ER acts on erythrocyte-specific genes that differ from known v-ErbA target genes (M.von Lindern, L.Boer, O.Wessely, M.Parker and H.Beug, submitted). We now demonstrate that the GR is an important regulator of erythropoiesis, whose function is mimicked by v-ErbA.

In contrast to the ER, the GR has been known for some time to affect erythropoiesis in mammals. Glucocorticoids were shown to enhance the formation of erythroid colonies *in vitro* (Golde *et al.*, 1976; Dainiak *et al.*, 1980; Billat *et al.*, 1982). In the presence of glucocorticoids, lower concentrations of erythropoietin were required to induce maximal erythroid cell proliferation (Udupa *et al.*, 1986). *In vivo*, glucocorticoids can restore normal erythropoiesis in pediatric aplastic anemia (reviewed in Liang *et al.*, 1994) and in Diamond-Blackfan anemia (Zito and Lynch, 1977; Chan *et al.*, 1982a,b). Treatment of non-anemic patients with the synthetic GR ligand prednisone was shown to result in increased erythropoiesis (Amylon *et al.*, 1986). These findings suggest that the ligand-activated GR enhances the proliferative capacity of erythroid progenitors. In addition, glucocorticoids were shown to induce γ - to β -globin gene switching (Zitnik *et al.*, 1995). Finally, glucocorticoids inhibit drug-induced hemoglobin accumulation in Friend virus-transformed mouse erythroleukemic cell lines (Tsiftoglou *et al.*, 1986; Kaneda *et al.*, 1995). The mechanism proposed for this block of differentiation is interaction of the GR with the erythroid transcription factor GATA-1 and subsequent repression of GATA-1 function (Chang *et al.*, 1993).

Here we present evidence that the GR is absolutely required for sustained proliferation and differentiation arrest of primary erythroid progenitors. To be active, the GR has to cooperate with the ligand-activated receptor tyrosine kinases c-ErbB and c-Kit. It also requires an activated ER for full response. This GR-induced progenitor self-renewal occurs mainly through transactivation of target genes containing GREs. Firstly, the partial GR antagonist RU486, which can induce nuclear translocation of the GR but not transactivation, acts as a pure antagonist

in erythroid cells, suggesting that repression of target genes is not sufficient to explain GR function. Secondly, a mutated ER that binds to a GRE instead of an estrogen response element (ERE), can fully replace GR function if activated by estradiol (E2). Subsequent mutation of the AF-2 domain in this mutated ER renders it totally inactive. This suggests that recruitment of the conserved AF-2 transactivation domain to the promoter of important GR target genes via GREs suffices for proper GR function in these cells. One such target gene may be *c-myb*, since its transcription is induced rapidly by the ligand-activated GR and the activated Δ c-Myb protein can replace GR function in erythroblasts.

Results

Dexamethasone is required for long term self-renewal of avian erythroblasts

Outgrowth and long-term proliferation (self-renewal) of avian erythroid progenitors was shown earlier to be dependent on the c-ErbB ligand TGF- α , the c-Kit ligand stem cell factor (SCF) and E2. In addition, the media had to contain chicken and fetal calf serum batches selected to support this long-term proliferation. If such sera were subjected to a combined charcoal-Freon treatment, their ability to sustain self-renewal was impaired. This suggested that (an) additional factor(s) were required to induce self-renewal (Steinlein *et al.*, 1995).

To identify such additional factors, we first tested various steroid hormone receptor ligands since the depletion method chosen efficiently removes lipophilic compounds. Freshly prepared chick bone marrow cells were seeded into medium containing charcoal-Freon-treated fetal calf and chicken sera plus SCF, TGF- α and E2. Outgrowth was monitored by daily counting. The cells were able to proliferate for ~7 days, but then became stationary (Figure 1A), indicating that these conditions support the transient proliferation of SCF progenitors (Hayman *et al.*, 1993) but not long-term self-renewal. In contrast, addition of dexamethasone (Dex), a synthetic GR ligand, as well as hydrocortisone induced the proliferation of bone marrow cells for at least 27 days (Figure 1A). Interestingly, the natural GR ligand hydrocortisone was fully active at physiological concentrations (10^{-10} M, data not shown). Aldosterone and dihydroxytestosterone were only active at non-physiological concentrations sufficient to activate the GR. This indicated that long-term self-renewal of SCF/TGF- α progenitors required activation of the endogenous GR.

To demonstrate that the GR was indeed expressed in normal erythroid progenitors, erythroblasts grown in the presence of Dex for 20 days were subjected to Western blot analysis using a rabbit antiserum to human GR cross-reactive with the chicken GR (see Materials and methods). As expected, the cells clearly expressed the 94 kDa GR protein (Figure 1B, lane 2). Other avian erythroid cell types, such as 4-day-old SCF progenitors and the erythroblast cell line HD3 also expressed the GR at clearly detectable levels. Chicken embryo fibroblasts were used as a positive control.

To demonstrate that the expressed GR was also functional in primary erythroid cells, transient transfections using a GRE-driven CAT reporter gene were performed.

Fig. 1. The outgrowth of self-renewing erythroid progenitors from bone marrow requires dexamethasone. (A) Freshly prepared chicken bone marrow cells were seeded into CFU-E medium containing Freon-charcoal-stripped fetal calf and chicken sera (stripped medium, see Materials and methods). They were supplemented with SCF and TGF- α plus either E2 alone (○) Dex alone (□) or both E2 and Dex (■). Cumulative cell numbers were determined as described earlier (Steinlein *et al.*, 1995). (B) Lysates of SCF progenitors (lane 1), TGF- α /SCF progenitors (lane 2), the erythroblast cell line HD3 (lane 3) and chicken embryo fibroblasts (lane 4) were processed for Western Blot analysis and analyzed using a GR-specific antibody. The position of the 94 kDa GR is indicated (arrow). (C) Cells were cultured as in (A), except that they were grown in CFU-E medium with untreated sera. They were supplemented with SCF, TGF- α and E2, plus either Dex (○) or ZK (□). (D) TGF- α /SCF progenitors were transfected with a GRE-containing CAT reporter construct and incubated for 24 h in media containing untreated sera either lacking added steroids or supplemented with 10^{-6} M ZK 112,993, RU486, dexamethasone or hydrocortisone. Relative CAT activities normalized to a control reporter construct (see Materials and methods) and to cell numbers are shown.

The transfected erythroblasts were incubated in media containing normal sera or normal sera supplemented with either Dex, hydrocortisone, a specific GR antagonist ZK 112,993 (ZK) or the partial antagonist RU486. CAT activities were then determined in cell extracts (Figure 1D). Dex and hydrocortisone induced reporter gene transcription, while both antagonists clearly prevented transcription. Furthermore, the relatively high levels of CAT activity found in cells exposed to media containing untreated sera only support our finding that the serum batches used indeed exhibit high levels of glucocorticoids.

To test whether the inability of charcoal-Freon-treated sera to support long-term proliferation of erythroid progenitors was due solely to depletion of GR ligands, the specific GR antagonist ZK was added to media containing untreated sera (expected to contain enough GR ligands to allow sustained self-renewal) plus SCF, TGF- α and E2. Bone marrow cells grown in this medium in the absence of the antagonist showed the expected normal outgrowth and long-term proliferation. In contrast, cells grown in

the presence of ZK ceased to proliferate around day 7, i.e. after the transient proliferation of SCF progenitors (Figure 1C). This indicated that ZK did not interfere with the proliferation of SCF progenitors, but effectively prevented the establishment of SCF/TGF- α progenitors (Hayman *et al.*, 1993). In addition, SCF/TGF- α progenitors pre-established in media containing normal sera and/or Dex and then exposed to ZK ceased to proliferate after 3–5 days (data not shown). Taken together, these data indicate that a ligand-activated GR is necessary for both the development and the long-term proliferation of SCF/TGF- α progenitors.

As shown above, E2 alone failed to support erythroblast outgrowth in charcoal-Freon-treated sera or in the presence of ZK. Interestingly, however, a combination of E2 and Dex gave rise to a much enhanced growth rate of the erythroid progenitors as compared with Dex alone (Figure 1A). This suggested that the GR and the ER have distinct, but cooperating functions in the proliferation of erythroblasts.

Table I. Frequency of colonies obtained in the absence or presence of dexamethasone by limited dilution cloning

Hormone mix	Cells seeded per well			
	20	100	500	2500
STED	8.3	4.0	3.9	n.d. ^a
	4.1	3.5	2.7	
STEZ		5.2		
	not done	not done	0.1 0 ^b	0.27 0.24

Frequency of immature colonies per 1000 cells seeded, determined 10 days after seeding. Data from two to three experiments using independent preparations of erythroid progenitors are shown. STED is SCF, TGF- α , E2 and Dex; STEZ is ZK 112,993 instead of Dex.

^aNot determined (n.d.) because the number of colonies was too high to be counted.

^bNo colonies could be grown from 30 000 cells seeded.

The target cell for GR action is a normal erythroid progenitor

Previously, we demonstrated by limiting dilution cloning that SCF, TGF- α , E2 and factors from chicken serum acted directly on single, c-Kit-expressing erythroid progenitors to induce their sustained proliferation (Steinlein *et al.*, 1995). The GR is expressed in many different hematopoietic cells, and GR ligands have profound effects on myeloid and lymphoid cells (Baxter and Forsham, 1972). It was necessary, therefore, to show that GR ligands stimulate proliferation by direct action on the erythroid progenitor and do not exert indirect effects via hematopoietic cells from other lineages.

For this, 3-day-old erythroid progenitor cultures [containing >95% immature, erythroid cells after purification (Dolznig *et al.*, 1995; Steinlein *et al.*, 1995)] were seeded at various densities into media containing SCF, TGF- α and E2. To these media were added either Dex (STED) or the GR antagonist ZK (STEZ). In the presence of STED, proliferating immature colonies developed at frequencies predicted from earlier data (Table I, Steinlein *et al.*, 1994). The *in vitro* life span of these clones was variable, but some clones attained 25 and more generations (data not shown). In contrast, no sustained outgrowth of erythroid cells could be observed in STEZ. The 20- to 50-fold reduced number of small colonies obtained in STEZ at day 10 failed to proliferate further and essentially all colonies had differentiated or died by day 13. These results clearly demonstrate that the GR ligands act on the normal erythroid progenitor to induce sustained proliferation and that no other cell types affected by Dex are required.

The GR requires cooperation with receptor tyrosine kinases to arrest erythroid differentiation

A prerequisite for any cell undergoing sustained proliferation is that it does not irreversibly enter a terminal differentiation program. In erythroblasts, differentiation induction not only drastically changes the expression of erythroid-specific genes, but also alters control of the cell cycle (Dolznig *et al.*, 1995; Müllner *et al.*, 1996). It was of interest, therefore, to determine if and how the ligand-activated GR would alter these aspects of the red cell differentiation program.

To induce differentiation of primary erythroid pro-

genitors, self-renewal factors (growth factors plus steroids) are replaced by differentiation factors [erythropoietin (Epo) and insulin, see Materials and methods]. Under these conditions, the differentiating cells accumulate hemoglobin and undergo 4–5 cell divisions within 2–3 days. Thereafter, they become post-mitotic, but continue to accumulate hemoglobin for another 2 days (Dolznig *et al.*, 1995). Thus, differentiating cells can be distinguished from self-renewing ones by measuring (i) their cumulative increase in cell number and (ii) the hemoglobin content per cell (O.Wessely, G.Mellitzer, M.von Lindern, A.Levitsky, A.Gazit, I.Ischenko, M.J.Hayman and H.Beug, submitted).

Dex had only minor effects on erythroid progenitors differentiating in the presence of Epo/insulin (Figure 2A and B). Both in the presence and absence of Dex, cell numbers increased for 4 days and then became stationary (Figure 2A and B). The mature erythrocytes obtained at this time contained comparable, high hemoglobin levels. However, erythrocytes formed in the presence of Dex were somewhat less hemoglobinized in the presence of Dex (Figure 2B). Identical results were obtained by adding the Dex antagonist ZK to those samples lacking Dex (data not shown). These results indicate that the GR alone does not interfere significantly with terminal differentiation.

Above, we showed that the activated GR induced sustained proliferation in erythroid progenitors in cooperation with the ligand-activated c-Kit or c-ErbB (Figure 1 and data not shown). Thus, the question arose as to whether or not the GR would require a similar cooperation with receptor tyrosine kinases also for differentiation arrest. To study this, we chose c-Kit, since activation of this receptor does not inhibit differentiation on its own. Rather, the c-Kit ligand SCF retards the erythroid differentiation program when added to cells in the presence of Epo/insulin (O.Wessely, G.Mellitzer, M.von Lindern, A.Levitsky, A.Gazit, I.Ischenko, M.J.Hayman and H.Beug, submitted). Under these conditions, erythrocytes were only formed at day 8. Within this time period, the cells underwent 8–9 cell divisions before becoming post-mitotic. Hemoglobin levels were low at day 4 but reached high levels similar to those in the absence of SCF at day 8 (Figure 2A and B). Surprisingly, Dex completely inhibited differentiation when added together with SCF plus Epo/insulin. The cells continued to proliferate throughout the experiment and retained low levels of hemoglobin as well as an erythroblast morphology in cytopins (Figure 2A and B and data not shown). Thus, ligand-activated GR can arrest differentiation, but this function absolutely requires cooperation with c-Kit.

Our data from the proliferation assays (Figure 1A) indicated that Dex may also cooperate with E2 in erythroid self-renewal. To study if a similar cooperation of the two hormones would also be operative in differentiation, E2, Dex and their respective antagonists ICI 182,780 (abbreviated as ICI) and ZK were added to cells differentiating in SCF plus Epo/insulin in all possible combinations. The use of these antagonists was necessary since anemic serum could not be stripped without loss of Epo activity. After 8 days, cells were cytocentrifuged onto slides and stained with histological dyes plus neutral benzidine to reveal hemoglobin (Figure 2C). The results clearly showed that a cooperation of the ER and the GR was required for

Fig. 2. Differentiation arrest of normal erythroid progenitors requires the cooperation between c-Kit and both the GR and the ER. Purified, 3-day-old SCF progenitors (Dolznig *et al.*, 1995) were seeded into Eptest medium plus anemic serum, insulin and E2. To this, ZK (■), Dex (□), SCF plus ZK (●) and SCF plus Dex (○) were added and cell numbers determined daily. Cumulative cell numbers obtained are depicted in (A). At the times indicated (A, arrows), hemoglobin was determined in aliquots of the cultures (Kowenz *et al.*, 1987) and plotted normalized to cell numbers (B, symbols above the bars refer to culture conditions mentioned in A). (C) Cells were induced to differentiate as described above in the presence of Epo, insulin, SCF and all possible combinations between E2, its antagonist ICI, Dex and its antagonist ZK. After 8 days, cytospin preparations of the different aliquots were stained with neutral benzidine plus histological dyes and images taken with a CCD camera (Photometrics), using a blue filter (480 nm) to reveal hemoglobin (Beug *et al.*, 1982, dark staining). Images were processed with Adobe Photoshop software.

the differentiation arrest. Only the culture receiving Dex plus E2 consisted mainly of immature erythroblasts at day 8 (Figure 8C, panel E2/Dex). In contrast, neither Dex in the presence of the ER antagonist nor E2 in the presence of the GR antagonist was able to arrest differentiation, as evidenced by the appearance of mature erythrocytes. These results indicate that the GR requires cooperation with the ER as well as with c-Kit to cause a full differentiation arrest.

Finally, we asked whether the GR would cooperate with c-ErbB in a similar fashion as with c-Kit. c-ErbB-expressing SCF/TGF- α progenitors (Hayman *et al.*, 1993) were induced to differentiate in the presence of TGF- α plus Epo/insulin. In addition, the cells received either Dex plus E2 or the two antagonists, ICI and ZK. In the presence of the antagonists, the ligand-activated c-ErbB was unable to arrest differentiation, allowing slow, but eventually terminal differentiation of the cells. In the presence of Dex plus E2, however, differentiation was tightly arrested by the c-ErbB ligand, very similar to the cooperation with c-Kit (data not shown). In contrast, Dex failed to cooperate with the ligand-activated insulin-like growth factor (IGF)-1 receptor, another tyrosine kinase expressed in our cells (data not shown). This indicates, that the GR cooperates with c-ErbB and c-Kit, but not with all receptor tyrosine kinases.

The partial GR antagonist RU486 acts as a pure antagonist in erythroid progenitors

The above results raise the obvious question as to the mechanism by which the GR exerts its function in erythro-

blasts. Two main mechanisms for how the ligand-activated GR regulates the transcription of target genes action have been proposed. Firstly, the GR transactivates through its AF-2 domain after binding to a GRE. Secondly, the GR can interfere with gene expression through interaction with other transcription factors, a process that requires neither DNA binding nor an intact AF-2 domain (transrepression; Heck *et al.*, 1994; Beato *et al.*, 1995). Agonists like Dex or hydrocortisone activate both functions. In contrast, the partial Dex antagonist RU486, at 10^{-6} M, fails to inhibit Dex-induced transrepression. In contrast, it completely blocks Dex-induced transactivation by the GR via its AF-2 domain under these conditions (Jonat *et al.*, 1990).

These facts prompted us to analyze the effect of RU486 on self-renewal and differentiation of erythroid progenitors. SCF/TGF- α progenitors were induced to differentiate in the presence of Epo/insulin, E2, SCF and either Dex, the pure antagonist ZK or RU486. Proliferation and differentiation were assayed as above by daily cell counting and determination of the hemoglobin content per cell at day 8. As observed before, cells cultured in Dex continued to proliferate throughout the experiment and accumulated little hemoglobin, while addition of ZK resulted in a stationary culture, complete maturation and high hemoglobin levels (Figure 3A and B). Interestingly, RU486 behaved indistinguishably from ZK. Cells in RU486 ceased to proliferate at day 8, like those in ZK, and accumulated the same high hemoglobin levels. Similarly to ZK (see Figure 1B), RU486 also completely

Fig. 3. The partial GR antagonist RU486 acts as a pure antagonist in avian erythroblasts. Purified, 3-day-old SCF progenitors were induced to differentiate as described in the legend to Figure 2, receiving Epo, insulin, SCF, E2 and either Dex (●), ZK (○) or RU486 (△) all at 1×10^{-6} M. Cell proliferation was monitored as described above (A) and hemoglobin content (B) was assayed at day 8 (A, dotted line plus arrow). Note that cells cease to proliferate and accumulate hemoglobin in RU486 as in ZK but proliferate without hemoglobin accumulation in Dex.

inhibited the outgrowth of cells from bone marrow in media containing SCF, TGF- α , E2 and untreated sera (data not shown). These results suggested that transrepression by GR interaction with other transcription factors (e.g. GATA-1, Chang *et al.*, 1993) is not the main mechanism operative in erythroid cells, leaving transactivation via specific DNA binding as another possible mechanism.

A mutated ER binding to a GRE can functionally replace the GR

If transactivation of genes containing a GRE is essential for the function of the GR in erythroid cells, a mutated ER that recognizes a GRE instead of an ERE could possibly replace the GR. DNA binding specificity is determined mainly by the P-box in the DBD. In the ER construct HE82, the P-box sequence of the ER was changed to the corresponding sequence in the GR (E203G, G204S and A207V). As a result, this steroid hormone receptor transactivated GRE-containing reporter genes upon activation by E2 (Mader *et al.*, 1989). If specific DNA binding to a GRE and an intact AF-2 domain are essential for the function of the GR in erythroid cells, an E2-activated HE82 protein should be able to substitute for the GR. To test whether the AF-2 domain is essential, the C-terminal part of HE82 was exchanged with the homologous mouse sequences that contained a L543A, L544A mutation, shown to inactivate the AF-2 function of the ER (Danielian *et al.*, 1992). As a control, we used the wild-type human ER (HEO).

The three receptors HE82, HEO and HE82/LALA were expressed in primary bone marrow cells using suitable retrovirus vectors (see Materials and methods). Mass cultures were established and expression of the constructs in the erythroblast cultures was verified by Western blot analysis. Cells infected with the HE82-, HE82/LALA- or HEO-containing vectors expressed the expected 66 kDa proteins, while cells infected with an empty vector did not (data not shown). To show that the stably expressed, exogenous receptors had the expected transactivation ability, the respective primary erythroblasts were transiently transfected with a GRE-driven CAT reporter gene. Cells were treated with Dex plus ICI, E2 plus ZK or with both antagonists for 24 h, and cell extracts were analyzed

for CAT activity (Figure 4A). As expected, all constructs failed to induce reporter gene transcription in the presence of both antagonists. Cells expressing the HEO or the AF-2-deficient HE82/LALA mutant showed reporter gene transcription only in response to Dex plus ICI (by activating the endogenous GR) but not in response to E2 plus ZK. This confirms that both HEO and HE82/LALA are unable to transactivate GRE-containing reporters. In contrast, HE82-expressing erythroblasts exhibited reporter gene transcription in response to both E2 (via the exogenous HE82) and to Dex (via the endogenous GR).

Next, we tested the ability of these mutant receptors to induce sustained proliferation of erythroblasts in the absence of Dex. For this, cells were cultured in SCF, TGF- α , E2 and either Dex or its antagonist ZK. Cumulative cell numbers were determined by daily counting (Figure 4B). As expected, erythroblasts expressing the HEO became stationary in the absence of Dex after 5 days. In contrast, cells expressing HE82 continued to proliferate throughout the experiment, regardless of the presence or absence of Dex. This indicated that the ER mutant with GRE binding activity was indeed able to substitute for a ligand-activated GR. Interestingly, the HE82/LALA mutant defective for AF-2-dependent transactivation was unable to replace the endogenous GR: cells treated with ZK ceased to proliferate in a similar way to the HEO-expressing control cells (Figure 4B). A control construct harboring the intact mouse AF-2 domain instead of the human one (see Materials and methods) behaved identically to HE82. These results indicate that specific DNA binding and a functional AF-2 domain are necessary for self-renewal induction by the GR in erythroblasts.

Finally, we analyzed whether the exogenous mutant ERs could replace the GR in its ability to cause a differentiation block in the presence of an active ER. Cells expressing the respective ER mutants were cultured in Epo/insulin plus SCF, either receiving E2 plus ZK or E2 plus Dex. Eight days after differentiation induction, cytospin preparations stained for hemoglobin were analyzed as described above (Figure 4C). Erythroblasts over-expressing HEO showed the same phenotype as control erythroid progenitors transfected with empty vector: cells were fully mature after E2 plus ZK treatment but proliferated as immature cells in the presence of E2 plus Dex. In contrast, the HE82-expressing cells retained their immature, proliferating phenotype regardless of whether Dex or its antagonist was added. This indicated that an E2-activated HE82 not only promotes long-term proliferation, but also efficiently arrests differentiation. Inactivation of AF-2 in the HE82/LALA mutant again rendered the construct inactive: erythroblasts expressing HE82/LALA terminally differentiated in E2/ZK (Figure 4C).

These data clearly show that binding to a GRE in combination with a functional AF-2 is necessary and sufficient for the function of the GR in erythropoiesis. Together with the data obtained with the partial antagonist RU486, the data strongly suggest that transactivation is the major mechanism by which the GR functions in avian erythroblasts.

Identification of c-myb as a potential target gene for the GR in erythroblasts

The above results indicated that the GR induced self-renewal mainly via transactivation of unknown target

Fig. 4. Proliferation stimulation and differentiation arrest by the GR requires specific DNA binding and an intact AF-2 domain. **(A)** Erythroid progenitors expressing the wild-type human ER (HEO), a human ER which binds to a GRE instead of to an ERE because of respective P-box mutations (HE82) and the latter construct carrying a mutated, inactive AF2 domain (HE82/LALA) were co-transfected with a GRE-containing CAT reporter construct and incubated with ICI plus ZK (black bars), ICI plus Dex (open bars) or E2 plus ZK (stippled bars) for 24 h. Relative CAT activities normalized to a control reporter construct (see Materials and methods) and to cell number are shown. **(B)** Cells from the same clones as shown in **(A)** were grown in CFU-E medium containing SCF, E2 and either Dex (□) or ZK (▲). Cells were counted at the times indicated and cumulative cell numbers determined as in Figure 1. **(C)** The erythroblast clones containing HEO, HE82 or HE82/LALA were induced to differentiate as above in Epo, insulin, SCF, E2 and either Dex (E2/Dex) or ZK (E2/ZK). Eight days after seeding, cells were cytocentrifuged, stained and images taken as described in the legend to Figure 2.

genes. To identify such genes, normal erythroid progenitors were withdrawn from all factors (receptor tyrosine ligands and steroid hormones) for 12 h, a time sufficient to allow degradation of most existing mRNA pools, but too short to induce significant apoptosis (see Materials and methods). Thereafter, cells were restimulated either with Dex or with ZK, alone or together with SCF. After incubation for 2, 12 and 24 h, cells were processed for Northern blot analysis. As first candidates, we analyzed the expression of several genes known or suspected to play a role in the regulation of erythroid proliferation and differentiation (Figure 5A). Blots were quantified by densitometric analysis (Figure 5B). *c-myb* was the only gene identified which was rapidly up-regulated by Dex. Already 2 h after re-stimulation with Dex, *c-myb* mRNA levels had increased 2- to 3-fold. Thereafter they slowly decreased, but stayed elevated with respect to the *c-myb* levels observed in cells treated with ZK. Interestingly, this up-regulation of *c-myb* mRNA by Dex occurred regardless of the presence or absence of SCF. In contrast, levels of α -globin mRNA remained low after restimulation with Dex, but were up-regulated with slow kinetics after addition of ZK (Figure 5B). Finally, *c-jun* was up-regulated in response to SCF, but not influenced by Dex, while GATA-1 was unaffected by Dex or SCF except at late time points (24 h, Figure 5B).

We then asked, if this Dex-induced up-regulation of *c-myb* mRNA levels (and the other changes in expression observed) occurred at the transcriptional level. For this, nuclei were prepared from cell preparations similar to those used for the Northern blot analysis and were processed for nuclear run-on analysis. The results obtained were similar to those of the Northern blot analysis, showing a 5- to 10-fold up-regulation of *c-myb* gene transcription, again independently of the presence or absence of SCF (data not shown). Our data suggest, therefore, that *c-myb* may be a target gene for the ligand-activated GR in primary erythroblasts.

An activated Myb protein (Δc -Myb) can substitute for the GR in self-renewal induction

Having identified *c-Myb* as a potential target subject to transcriptional activation by the GR, we asked whether or not *c-Myb* would actually function in induction of self-renewal. Since *c-Myb* should act downstream of the GR, a constitutively active version of *c-Myb* should be able to partially or even fully replace the biological activity of the GR, i.e. stimulate prolonged proliferation and arrest differentiation in the presence of a GR antagonist. The *c-Myb* protein is present in an inactive conformation in most cells and has to be activated by unknown mechanisms. Therefore, we used an activated *c-Myb* protein in

Fig. 5. Identification of c-Myb as a potential target gene of GR function in erythroblasts. Ten-day-old erythroid progenitors grown in SCF, TGF- α , E2 and Dex were withdrawn from all factors for 12 h (stripped medium plus 40 ng/ml IGF-1). Thereafter, the cells were restimulated with either Dex or its antagonist ZK for the times indicated, in the presence or absence of SCF. Aliquots containing equal numbers of cells were then processed for Northern blot analysis with the probes indicated. (A) Shows the actual blots obtained. In (B), results from a quantitative evaluation of the blots using a Phosphorimager are shown after normalization to the 28S signal. These results could be confirmed in three independent experiments.

which an N-terminal domain carrying phosphorylation sites and a C-terminal inhibitory domain thought to be important for keeping c-Myb in an inactive state had been removed (for a recent review, see Introna *et al.*, 1994).

Bone marrow cells were infected with a retrovirus expressing the Myb EEA protein (referred to as Δ c-Myb) together with the human epidermal growth factor receptor (EGF-R)/c-ErbB as a selection marker (M.Zenke and H.Beug, unpublished). As a control, parallel cultures were infected with a virus expressing human c-ErbB only (Khazaie *et al.*, 1988). Transformed colonies selected in Methocel in the presence of TGF- α , E2 and Dex were isolated, grown into mass cultures and tested for the expression of the 56 kDa Δ c-Myb protein by Western blot.

To analyze whether the presence of Δ c-Myb abrogates the strict requirement for Dex, cells of a well growing EGF-R/myb EEA clone were seeded in medium containing SCF, TGF- α , ICI (to block the function of the ER) and either Dex or its antagonist ZK. As control cells, a clone expressing the human EGF-R only was employed. Cells were counted daily and cumulative cell numbers determined (Figure 6A). In the presence of an active Δ c-Myb (Figure 6A, EGF-R/ Δ c-Myb), the cells proliferated almost as well in the presence of ZK as in the presence of Dex. In contrast, control cells expressing the EGF-R proliferated as expected when supplemented with Dex. In the presence of ZK, however, cell proliferation ceased after a few days and the cells disintegrated (Figure 6A, control). These results demonstrate that an active Δ c-Myb protein can replace the function of the GR required for sustained proliferation.

To analyze whether Δ c-Myb would also arrest differentiation in the absence of an activated GR, several EGF-R/ Δ c-Myb-expressing clones were induced to differentiate in Epo/insulin, SCF and E2, adding either Dex or its antagonist ZK. After 7 days, stained cytospin preparations of the two cell populations were prepared and evaluated. In a representative Δ c-Myb-expressing clone (#E2), differentiation was arrested both in the presence and absence of Dex: both cultures contained a large majority of erythroblasts (Figure 6B, right panels). In contrast, control clones expressing EGF-R/c-ErbB only required Dex for their arrest of differentiation and fully matured into erythrocytes in the presence of the Dex antagonist ZK (Figure 6B, left panels).

In conclusion, these data show that Δ c-Myb abrogates the requirement for an activated GR. Therefore, c-Myb may indeed act downstream of the GR, representing one potentially important GR target gene in sustaining proliferation and inhibiting differentiation.

Discussion

We demonstrate here that the GR is essential for the long-term proliferation of avian erythroid progenitors. A ligand-activated GR was required (i) for the development of SCF-dependent progenitors into self-renewing SCF/TGF- α -dependent progenitors, (ii) for the sustained proliferation of established SCF/TGF- α -dependent progenitors and (iii) for the arrest of erythroid differentiation essential for long-term self-renewal (Hayman *et al.*, 1993; Steinlein *et al.*, 1995). However, the activated GR had

Fig. 6. An activated c-Myb protein can replace the GR in stimulation of erythroid progenitor proliferation and differentiation arrest. (A) Primary erythroblasts expressing either a retrovirus co-expressing Δ c-Myb and the human EGF-R (EGF-R/ Δ c-Myb, right panels) or control cells expressing the EGF-R only (left panels; control) were grown in SCF, TGF- α , E2, as well as in the presence of Dex (□) or ZK (▲). Cumulative cell numbers were determined as described in the legend to Figure 1. (B) Cells from the same clones as in (A) were induced to differentiate in medium containing Epo, insulin, SCF, E2 and either Dex (E2/Dex) or ZK (E2/ZK). Eight days after differentiation induction, cells were cytocentrifuged, stained and images taken as described in the legend to Figure 2.

little biological effect on its own, being totally dependent on the simultaneous activation of c-Kit or c-ErbB. In many cell types, the activated GR acts by inhibiting the activity of transcription factors interacting with the GR (see Introduction). In our system, however, the GR acts mainly through the activation of target genes, depending on the specific binding to a GRE and on an intact AF-2 transactivation domain. One potentially important target gene of the GR is *c-myb*.

How does the GR cooperate with receptor tyrosine kinases and other nuclear hormone receptors?

A striking feature of GR action in erythroblast self-renewal was its complete dependence on ligand-activated receptor tyrosine kinases such as c-Kit or c-ErbB. When Dex or its antagonist ZK were added to erythroid progenitors differentiating in Epo/insulin alone, they had practically no effect. Only after activation of c-Kit or c-ErbB by their

ligands SCF or TGF- α did Dex induce stable self-renewal under otherwise identical differentiation conditions, while the GR antagonist ZK allowed terminal differentiation to occur.

The second conclusion to be drawn from these observations is that the endogenous receptor tyrosine kinases c-Kit and c-ErbB require a ligand-activated GR to induce self-renewal. On their own, these kinases stimulate proliferation but are unable to arrest erythroid differentiation. Thus, cells stimulated by SCF or TGF- α in the absence of an active GR undergo an elevated number of cell divisions, but eventually form mature erythrocytes.

What is the mechanism by which the GR cooperates with the receptor tyrosine kinases ErbB and c-Kit? This cooperation depends, to some extent, on the type of tyrosine kinase, since other tyrosine kinases such as the IGF-1 receptor, the insulin receptor and Jak2 activated by the Epo receptor are unable to cooperate with the GR (O.Wessely and H.Beug, unpublished observations). One potential mechanism involved is direct phosphorylation of the GR by kinases activated by signal transduction from the receptor. In pilot experiments, Dex similarly induced transcription of a transiently transfected, GRE-driven reporter construct both in the presence and absence of a ligand-activated c-Kit (data not shown). In addition, *c-myb* mRNA expression was up-regulated rapidly both in the presence and absence of a ligand-activated c-Kit (Figure 5). This suggested that signal transduction from c-Kit (which could result in phosphorylation of the GR) did not influence transactivation of GR target genes in erythroid progenitors. The experiment does not rule out the possibility that direct phosphorylation is important in the context of natural, more complex promoters, where phosphorylation may be required to trigger interaction with co-activators or other cooperating transcription factors. Another possible scenario for the way in which c-Kit and the GR cooperate could be that the protein products of GR target genes and proteins activated directly or indirectly through c-Kit signaling (such as c-Jun) cooperate to induce self-renewal. This idea is strengthened by the fact that *c-jun* mRNA was clearly up-regulated by SCF, but was not affected by ligand activation of the GR.

The requirement of the GR to cooperate with receptor tyrosine kinases is paralleled strikingly by the cooperation between v-ErbA and endogenous or exogenous normal or oncogenic tyrosine kinases. v-ErbA is a heavily mutated version of the thyroid hormone receptor (Sap *et al.*, 1986), exhibiting an altered DNA binding specificity (Bonde *et al.*, 1991; Smit-McBride and Privalsky, 1994). We recently have demonstrated (A.Bauer, E.Ulrich, M.Andersson, H.Beug and M.von Lindern, submitted), that v-ErbA is totally inactive in erythroblasts unless these contain an active tyrosine kinase, either oncoproteins like v-ErbB or v-Sea (Hayman and Beug, 1992) or ligand-activated, endogenous c-Kit or c-ErbB (Beug *et al.*, 1994). In cooperation with such kinases, v-ErbA abrogates the requirement of activated steroid hormone receptors like the ER or GR, both for self-renewal and differentiation arrest. Like the GR, v-ErbA is unable to cooperate with the IGF-1 or insulin receptors (M.von Lindern, unpublished observations). This strongly suggests that the GR represents the endogenous regulator, whose biological function is replaced by the retroviral oncoprotein v-ErbA

in leukemogenesis. At present, however, we do not know at which level this replacement of GR function by v-ErbA occurs and whether similar or different mechanisms are involved.

Interestingly, two other nuclear hormone receptors, the retinoid acid (RAR) and the TR, also exhibit pronounced effects on erythroid differentiation. In contrast to the GR, however, TR and RAR accelerated erythroid differentiation when activated by their respective ligands (Schroeder *et al.*, 1992). This interplay is also observed at the molecular level. Dex, as shown here, up-regulates transcription of *c-myb*, while retinoid acid rapidly down-regulates it (Smarda *et al.*, 1995). Thus, cross-talk between various members of the nuclear hormone receptor family may be responsible for regulating the balance between proliferation and differentiation in erythroid progenitors.

Mechanism of GR action in erythroblasts: transactivation or transrepression?

A major known activity of the GR in hematopoietic cells is the induction of programmed cell death. Glucocorticoids induce apoptosis in lymphocytes, monocytes and eosinophils as well as in a variety of leukemic cell lines (see Helmberg *et al.*, 1995, and references therein). Also, the GR strongly affects erythroid differentiation in Friend erythroleukemia cells (Chang *et al.*, 1993). However, no defects in erythropoiesis affecting embryo viability were observed in mice lacking a functional GR (Cole *et al.*, 1995). To date, a detailed analysis of the erythroid compartment has not yet been completed.

The proposed common mechanism by which the GR induces these effects is transrepression, involving interactions with NF κ B family members, AP-1 and GATA-1 (see Introduction). Here we present two lines of evidence that proliferation induction and differentiation arrest caused by the GR mainly depends on mechanisms involving transactivation. Firstly, the partial GR antagonist RU486, which causes nuclear translocation of the GR, but cannot activate AF-2, was unable to sustain self-renewal or to block differentiation in erythroid progenitors. In contrast, aldosterone, which binds to and activates transcription by the GR but fails to activate transrepression (Heck *et al.*, 1994), strongly supported self-renewal, acting like a GR agonist (data not shown). These results strongly argue that transrepression is not important or at least not sufficient for the effects of the GR on primary erythroid cell self-renewal.

Secondly, we obtained direct evidence that DNA binding and transactivation are important for the function of the GR in erythroid cells. We used a mutant ER (HE82) containing three point mutations (E203G, G204S and A207V) in its P-box, causing it to bind to a GRE instead of an ERE (Mader *et al.*, 1989) and the same ER variant containing a non-functional AF-2 domain [HE82/LALA; carrying the mutations L543A and L544A (Danielian *et al.*, 1992)]. Overexpression of HE82 in erythroblasts rendered the cells independent of Dex for proliferation and differentiation arrest, while a control wtER construct expressed in the same cells was not able to do so. The only difference between the mutant and wild-type receptors is their DNA binding specificity. Therefore, specific binding of a steroid hormone receptor to a GRE was obviously a necessary event in self-renewal induction. In addition,

a functional AF-2 domain is required, since the HE82/LALA mutant was unable to replace the function of the endogenous GR. Obviously, the high degree of sequence conservation of the AF-2 domain between the ER and the GR allowed the ER to function as a GR, if recruited to target genes containing GREs.

Sequence-specific DNA binding and the presence of an intact AF-2 domain are not only necessary for transactivation by the GR, but also for the alterations in chromatin structure. Our experiments do not allow us to conclude which of these processes is essential for the function of the GR in erythropoiesis. In this respect, it is intriguing that glucocorticoids are essential for the development of SCF progenitors into SCF/TGF- α progenitors that are able to undergo long-term self-renewal. This process requires both time (>7 days) and several cell divisions. In addition, it most likely involved changes in many genes rather than an up-regulation of c-ErbB only. A similarly retarded response was observed in cells which expressed an exogenous mutant ER unable to replace GR function (HEO or HE82/LALA). Also in these cells, withdrawal from Dex resulting in the inactivation of the GR caused a proliferation arrest only 7–10 days after hormone withdrawal (Figure 4B). Therefore, the activated GR may cause erythroid progenitors to undergo epigenetic changes involving alterations in chromatin structure (Muchardt and Yaniv, 1993), thus establishing a developmental program required for stable self-renewal of erythroid cells.

Of course, our results do not exclude the possibility that transrepression (dependent on interactions between steroid hormone receptors and GATA-1, AP1, NF κ B or other factors; see Introduction) also occurs during induction of self-renewal. Such a mechanism probably plays no major role in the induction of cell proliferation and differentiation arrest by the GR. It might, however, affect later stages of erythroid differentiation, for instance hemoglobin accumulation in erythrocytes (see Figure 2B), since GATA-1 is responsible for up-regulation of globin gene expression (Minie *et al.*, 1992). A similar mechanism was found for the ligand-activated ER. In addition, the active ER also repressed other erythroid-specific genes (M.von Lindern, L.Boer, O.Wessely, M.Parker and H.Beug, submitted). Thus, transrepression could play an important role in the observed cooperation between the ER and the GR in regulating the balance between proliferation and differentiation.

***c-myb*, an important GR target gene regulating self-renewal?**

The c-Myb protein is expressed in immature hematopoietic cells of the erythroid, myeloid and lymphoid lineages (for review, see Introna *et al.*, 1994). c-Myb is clearly important for hematopoiesis, since mice suffering targeted disruption of the *c-myb* gene (*c-myb* $-/-$ mice) die on around day 15 of embryonic development and lack all hematopoietic lineages except megakaryocytes (Mucenski *et al.*, 1991). In Friend murine erythroleukemia cells, constitutive overexpression of c-Myb can block hemoglobin accumulation in response to dimethyl sulfoxide (DMSO) (Clarke *et al.*, 1988). In the same cells, a truncated version of c-Myb lacking the transactivation domain accelerated DMSO-induced hemoglobinization (Weber *et al.*, 1990). Although expression of c-Myb is essential in hematopoiesis, little

is known about the regulation of c-Myb expression during normal erythroid differentiation except that it is down-regulated rapidly after differentiation induction of normal erythroid progenitors (Dolznig *et al.*, 1995). Furthermore, expression of c-Myb is regulated at both transcriptional and post-transcriptional levels, most likely involving multiple, cooperating factors (Thompson and Ramsay, 1995). We have demonstrated here that Dex rapidly elevated c-*myb* mRNA levels as well as the rate of c-*myb* gene transcription. The c-Myb promoter does contain sequences resembling GREs (P.Bartunek, personal communication). Experiments are in progress to determine whether c-Myb activation is due to direct binding of the GR to the c-Myb promoter and whether post-transcriptional/translational mechanisms are also involved.

If c-*myb* represents an essential target gene for the GR, an active c-Myb would be expected to act downstream of the GR in the pathway causing self-renewal, thereby replacing its biological activity. In contrast to c-Myb, which is inactive as a transcription factor in most cells, mutated oncogenic versions of c-Myb have been shown to be constitutively active (Grasser *et al.*, 1991; Hu *et al.*, 1991). This seems to be due to the loss of a C-terminal regulatory domain which represses transactivation by c-Myb unless removed by proteolysis (Grasser *et al.*, 1991) or rendered inactive by mutations in v-Myb (Introna *et al.*, 1994). We show here that an activated c-Myb, together with a ligand-activated c-ErbB, replaces GR function in self-renewal while c-ErbB alone requires cooperation with the GR to induce self-renewal. Thus, erythroblasts co-expressing c-Myb and the EGF-R proliferate as immature cells in the presence of TGF- α plus the GR-antagonist ZK. In contrast, control cells expressing c-ErbB only stopped proliferating under the same conditions and differentiated into erythrocytes, if differentiation factors (Epo/insulin) were also present.

Interestingly, the activation of c-Myb seems not to be sufficient. Rather, signals emanating from tyrosine kinase receptors and the continuous presence of E2 seem to be required as well. Firstly, EGF-R/ts- Δ c-Myb cells induced to differentiate in the absence of the c-ErbB ligand showed only a small retardation of differentiation at the permissive temperature (H.Beug, unpublished, see also Casini and Graf, 1995). Secondly, the block of differentiation by Δ c-Myb cannot be maintained in the absence of E2 (data not shown), again arguing that E2 and Dex have distinct functions in erythropoiesis.

In conclusion, the following model for GR function in self-renewal induction of erythroid progenitors can be proposed. Ligand activation of the GR causes transcriptional activation of c-Myb, which in turn contributes to proliferation stimulation and differentiation arrest of the erythroid progenitors. Both the GR and c-Myb itself may require cooperation with both receptor tyrosine kinases and an active ER, through mechanisms not yet understood. This model represents the first, still rather speculative, molecular explanation of how steroid hormone and tyrosine kinase receptors may cooperate in regulating the balance between proliferation and differentiation in committed hematopoietic progenitors. It also sheds light on the equally important question of how mutated versions of these two gene families may cooperate to grossly tip

this balance towards proliferation, with the outcome of fatal erythroleukemia.

Materials and methods

Viruses and cells

Chicken embryo fibroblasts (CEFs) expressing the retroviral constructs EGF-R/ Δ c-Myb (EEA-myb), HEO and HE82 were generated and used as described (Zenke *et al.*, 1990a; M.von Lindern, L.Boer, O.Wessely, M.Parker and H.Beug, submitted; M.Zenke and H.Beug, unpublished results). HEO and HE82 were a kind gift of M.Parker, ICRF, London. DNA fragments containing the open reading frame of these receptors were excised from the expression vector pJ3 (White *et al.*, 1987) and inserted behind the cytomegalovirus (CMV) promoter of the avian retroviral expression vector pCRN (Steinlein *et al.*, 1994). To generate HE82/LALA, a *Ssp*BI-*Bam*HI fragment of the HE82 containing the AF-2 domain was exchanged with the corresponding fragment of the MOR L543A/L544A mutant described previously (M.von Lindern, L.Boer, O.Wessely, M.Parker and H.Beug, submitted). As a control, we also generated a hybrid of the HE82 DNA binding domain fused to the murine wt AF-2 domain using similar procedures.

All fibroblasts were grown in standard growth medium (Graf, 1973). SCF progenitors and SCF/TGF- α progenitors were grown from the bone marrow of 3- to 10-day-old Spafas chicks as described previously (Hayman *et al.*, 1993; Schroeder *et al.*, 1993; Steinlein *et al.*, 1995). CFU-E medium containing Freon-charcoal-stripped fetal calf and chicken sera (used exclusively for the experiment shown in Figure 1) was prepared as described earlier (Steinlein *et al.*, 1995). With the exception of hydrocortisone, which was used at 10^{-11} M, all steroids (estradiol, dexamethasone) and the steroid antagonists ICI 182,780, ZK 112,993 (Mikulits *et al.*, 1995) and RU486 were used at a final concentration of 10^{-6} M.

Infection of primary erythroblasts with retroviruses

To infect erythroblasts, freshly prepared chicken bone marrow cells were co-cultivated for 2 days with mitomycin C-treated CEFs expressing the respective retroviral constructs (Fuerstenberg *et al.*, 1992). The cells were then either propagated further as a mass culture or seeded in CFU-E-Methocel containing avian SCF (100 ng/ml, Bartunek *et al.*, 1996), TGF- α (5 ng/ml, Promega), E2 and Dex. Colonies growing out from the virus-infected cells were isolated and expanded in CFU-E medium plus SCF, TGF- α , E2 and Dex (Hayman *et al.*, 1993).

Proliferation kinetics

To determine the growth kinetics of erythroblast mass cultures, cells were subjected to daily partial medium changes plus re-addition of fresh factors. They were kept at densities between 2 and 4×10^6 cells/ml by appropriate dilution, and aliquots were counted in an electronic cell counter (CASY-1, Schärfe-System, Germany). Cumulative cell numbers were determined as described in Fuerstenberg *et al.* (1992).

Limited dilution cloning

This assay was performed essentially as described earlier (Steinlein *et al.*, 1995). Briefly, purified 3-day-old SCF progenitors were seeded into 96-well plates at various concentrations (20, 100, 500 and 2500 cells per well) into CFU medium containing SCF, TGF- α , E2 and either Dex or its antagonists ZK 112993. Partial medium changes were performed every 2–3 days, taking care not to disperse or change the location of the colonies. At day 10, the numbers of immature, healthy colonies were counted. Results from the different dilutions were pooled and found to be essentially linear with cell numbers seeded. Data are expressed as the mean number of colonies obtained per 1000 cells seeded, based on counting of >240 wells.

Differentiation induction

Cells were washed twice in serum-free medium and seeded at $1-2 \times 10^6$ cells/ml into 35 mm dishes containing 2 ml of differentiation medium which contained untreated sera (Dolznig *et al.*, 1995). As a source of avian Epo, 3–5% high titer anemic serum was added; in addition, the cultures were supplemented with insulin (23 mU/ml corresponding to 1.4 nM). When indicated, avian SCF (100 ng/ml), 1×10^{-6} M E2, its antagonist ICI, Dex or its antagonist ZK were added. Cells were counted daily and aliquots removed for hemoglobin determination. The cells were maintained at densities between 2 and 4×10^6 cells/ml by daily

medium addition or partial medium changes plus re-addition of growth factors.

Analysis of differentiation by cell morphology and staining

Cells were cytocentrifuged onto slides and subsequently stained with histological dyes and neutral benzidine for hemoglobin as described in Beug *et al.* (1982). Images were taken using a CCD camera (Photometrics) and a blue filter (480 nm), so that mature cells (stained yellow to brownish) appear darkly stained. Images were processed with Adobe Photoshop.

Photometric hemoglobin assay

Aliquots (3×50 µl) of the cultures were removed and processed for photometric determination of hemoglobin as described (Kowenz *et al.*, 1987).

Transient transfection and CAT assay

SCF/TGF- α progenitors were transfected using the Lipofectamine™ reagents, according to the manufacturer's instructions. For transfection, 3.2 µg of reporter DNA constructs (pBL-2GRE-CAT2), a kind gift of Günther Schütz, Heidelberg, Germany and 10 µl of Lipofectamine™ reagents were mixed, pre-incubated and the complex added to 1×10⁷ cells. After incubation for 4 h, the cells were cultivated further in differentiation medium containing TGF- α , SCF and the respective steroids or their antagonists for 24 h. Following incubation, cytoplasmic extracts were prepared by three cycles of freeze-thaw in 0.25 M Tris-HCl (pH 7.5), and CAT assays were performed using a CAT enzyme-linked immunosorbent assay (Gorman *et al.*, 1982). Transfection efficiency was assessed by co-transfection of a reporter plasmid containing a Rous sarcoma virus promoter-driven β -galactosidase gene, using a chemiluminescence substrate (Tropix, Bedford, USA). CAT activities obtained were normalized to transfection efficiencies as well as to cell numbers.

Northern blot analysis

Normal erythroid progenitors (5–10×10⁶ cells per sample) were incubated in differentiation medium lacking all factors except 40 ng/ml IGF-1 (R&D) overnight. They were then stimulated for 2, 12 and 24 h with Dex or its antagonist ZK, in the presence or absence of 100 ng of avian SCF. RNA was then isolated according to published procedures (Chomczynski and Sacchi, 1987), with minor modifications. Cells were lysed in GITC buffer and NaAc, pH 4.0 was added to 25 mM. The solution was extracted with H₂O-saturated phenol, chloroform and isoamylalcohol. After precipitation with isopropanol, the pellet was dissolved in 10 mM Tris-HCl pH 7.0, 1 mM EDTA, 0.2% SDS, and proteinase K was added to 200 µg/ml. After 30 min incubation at 37°C, the solution was extracted with phenol:chloroform:isoamylalcohol (25:24:1) pH 7 and precipitated with ethanol. Then 10–20 µg of RNA was run on a formaldehyde-containing agarose gel and transferred to nylon filters (Gene Screen) using conventional procedures. Single-stranded DNA probes were radioactively labeled with ³²P by using an Oligolabelling Kit (Pharmacia) and hybridized at 65°C in 7% SDS, 0.5 M Na phosphate pH 7.0, 1 mM EDTA. Membranes were washed in 1% SDS, 40 mM Na phosphate at 65°C and autoradiographed. Probes used were: c-Myb, GATA-1, α -globin and c-Jun, described in Briegel *et al.* (1993) and Dolznig *et al.* (1995).

Run-on analysis

Cell preparations similar to those used for Northern blot analysis were harvested as above. Nuclei were isolated and processed for run-on analysis as described earlier (Zenke *et al.*, 1990b). Radioactively labeled RNA was hybridized to filters onto which total plasmid DNA containing the probes mentioned above had been blotted.

Western blot for GR expression

Various types of avian (Beug *et al.*, 1994) erythroblasts and human (M.von Lindern, L.Boer, O.Wessely, M.Parker and H.Beug, submitted) erythroblasts as well as Friend erythroleukemia cells (Patel and Lodish, 1987; as a positive control), were washed in 1× phosphate-buffered saline (PBS), harvested by centrifugation, lysed in 1% Triton-X-100, 100 mM NaCl, 50 mM Tris-HCl (pH 8.0) and analyzed by Western blot analysis as described (Crowe and Hayman, 1991). As an antibody to the GR, a rabbit antiserum to the mouse GR cross-reacting with the chicken protein (a kind gift of Professor Dr G.Schütz, Heidelberg, Germany) was used.

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