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Distinct downstream signaling mechanism between erythropoietin receptor and interleukin-2 receptor

Yasuko Yamamura[1,4], Yukio Kageyama[2], Tomoko Matuzaki[3], Makoto Noda[3] and Yoji Ikawa[1,4]

[1]Department of Biochemistry and [2]Department of Urology, Tokyo Medical and Dental University School of Medicine, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113, [3]Department of Viral Oncology, Cancer Institute, Japanese Foundation for Cancer Research, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170 and [4]Laboratory of Molecular Oncology, Tsukuba Life Science Center, The Institute of Physical and Chemical Research (RIKEN), 3-1-1 Koyadai, Tsukuba, Ibaraki 305, Japan

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Erythropoietin receptor (EPOR) and interleukin-2 receptor β chain (IL-2Rβ) belong to the same cytokine receptor superfamily and have highly conserved sequences in their intracellular signaling domain. However, common downstream signaling pathways of these receptors have not been demonstrated. In the present study, we introduced and expressed the murine EPOR in murine IL-2-, IL-3- and IL-5-dependent cell lines and analyzed their growth response to EPO. We found that the expression of EPOR induced EPO dependence in IL-3-dependent BAF-B03 and IL-5-dependent Y16 cells but not in IL-2-dependent CTLL-2 cells, although the EPOR-expressing CTLL-2 cell lines could bind and internalize EPO as efficiently as the BAF-B03-derived cell lines. Additional expression of AIC2B, a common signal transducer for IL-3R, IL-5R and GM-CSFR, made no difference to the EPO responsiveness of the EPOR-expressing CTLL-2 cell lines. These results suggest that the cellular components required for the transduction of EPOR signal and IL-2R signal are at least partially different, and this difference cannot be explained solely by the absence of AIC2B.

Key words: cytokine receptor family/downstream signaling pathways/EPO receptor/IL-2 receptor β chain/IL-3 receptor β subunits

Introduction

Erythropoietin (EPO) is a glycoprotein which mediates signals for proliferation and differentiation of erythroid progenitor cells. EPO binds to a cell surface receptor (EPOR) and thereby stimulates the growth of some cell lines (Sawyer *et al.*, 1987b; Spivak *et al.*, 1991), and the growth and differentiation of some other cell lines of erythroid lineage (Erslev, 1987). In spite of the importance of EPO in the regulation of erythropoiesis (Eschbach *et al.*, 1987), little is known about the mechanism of signal transduction following EPO-EPOR interaction.

The cDNAs for the human and murine EPOR have been cloned, and their structures have been elucidated (Jones *et al.*, 1990; D'Andrea *et al.*, 1989a). EPOR belongs to the cytokine receptor superfamily which includes the receptors for a number of cytokines and hormones such as interleukin-2 (IL-2), IL-3, IL-4, IL-5, IL-6, IL-7, granulocyte/macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), growth hormone (GH) and prolactin (PRL) (D'Andrea *et al.*, 1989b; Bazan, 1990; Cosman *et al.*, 1990). These receptors share some common structural features in that they all have the cysteine motif (four aligned cysteine residues) and the WS motif (WSXWS or Trp-Ser-X-Trp-Ser sequence) in their extracellular region and no kinase domain in the intracellular region. In addition, the receptors for EPO, IL-2, IL-3 and IL-4 share structural similarities particularly in their cytoplasmic regions, and this may suggest that a common downstream signaling pathway exists for these receptors (D'Andrea *et al.*, 1990). The lymphocyte-specific protein tyrosine kinase $p56^{lck}$ has been shown to form a stable complex with the IL-2 receptor β chain (IL-2R β) and probably to participate in IL-2R signaling (Hatakeyama *et al.*, 1991). Similarly, certain tyrosine kinases may play a role in signal transduction by EPOR, IL-3R and IL-4R.

Between EPOR and IL-2Rβ there exist two highly conserved segments, extracellular segment I (aligned amino acids 219-251) and cytoplasmic segment II (amino acids 310-357) (D'Andrea *et al.*, 1989b; Hatakeyama *et al.*, 1989b; Kono *et al.*, 1990). Segment II seems an essential domain for growth signaling in EPOR and IL-2Rβ (Hatakeyama *et al.*, 1989b; D'Andrea *et al.*, 1991). In fact, some IL-3-dependent cell lines have been reported to grow in the presence of EPO or IL-2 when the respective cDNA for EPOR or IL-2Rβ was exogenously introduced and expressed in the cells (Hatakeyama *et al.*, 1989b; Kono *et al.*, 1990; Li *et al.*, 1990; Mori *et al.*, 1990; Yoshimura *et al.*, 1990; D'Andrea *et al.*, 1991; Li and Baltimore, 1991). These observations suggest the presence of common growth signaling mechanisms for EPOR and IL-2Rβ. However, there have been no reports on similar studies using IL-2-dependent cell lines, and it is not clear whether EPOR can also use the same signaling pathway as IL-2R.

To address the above issue we introduced a murine EPOR cDNA, using retrovirus-mediated gene transfer, into a murine IL-2-dependent T cell line, CTLL-2, and investigated whether EPO can support the proliferation of these EPOR-expressing cells.

Results

Expression of cDNA for EPOR in IL-2-dependent (CTLL-2) and IL-3-dependent (BAF-B03) cells

To introduce a mouse EPOR cDNA into an IL-2-dependent mouse T cell line CTLL-2 (Cerottini *et al.*, 1974) and IL-3-dependent mouse pro-B cell line BAF-B03 (Hatakeyama *et al.*, 1989b), we first constructed a pLXSN

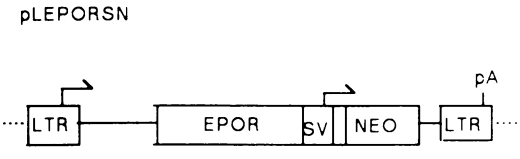

Fig. 1. Construction of retroviral vector used to express EPOR. The cDNA for mouse EPOR was inserted into retroviral vector, pLXSN (Miller and Rosman, 1989). LTR, the retroviral LTR that contains the retroviral promoter and enhancers; SV, SV40 early region promoter and enhancers; EPOR, mouse EPOR cDNA; NEO, neomycin resistance gene; pA, poly(A) site. Lines indicate retroviral sequences. Arrows denote the site of RNA transcription initiation.

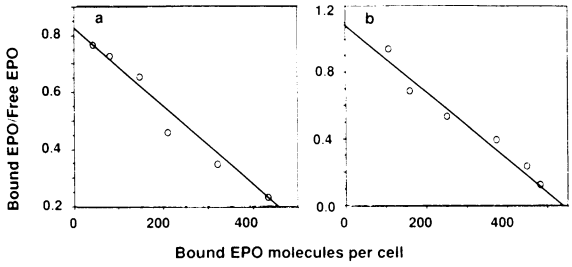

Fig. 2. Scatchard plot analyses of [^{125}I]EPO binding to IL-2-dependent CTLL-2 and IL-3-dependent BAF-B03 cells expressing EPOR. (**a**) CTLL-2-derived clone expressing EPOR, C/EPOR 12B3 and (**b**) BAF-B03-derived clone expressing EPOR, B/EPOR 12B4. Cells (5×10^6) were incubated with various concentrations of ^{125}I-labeled human EPO for 4 h at 10°C and specific binding was measured. Scatchard plot analyses of [^{125}I]EPO-specific binding were performed.

based retroviral vector, named pLEPORSN carrying a mouse EPOR cDNA (Kuramochi *et al.*, 1990) (Figure 1). This vector contains the neomycin resistance gene as a selectable marker (Miller and Rosman, 1989). We then transfected the pLEPORSN DNA into the PA317 packaging host, and selected a cell clone producing a high titer of the EPOR virus. We infected this virus into CTLL-2 and BAF-B03 cells, and selected the infected cells in medium containing an appropriate cytokine and G418 (1 mg/ml and 2 mg/ml, respectively).

We then examined the expression of EPOR on the surface of the EPOR virus infected cells by an [^{125}I]EPO binding assay. Scatchard plot analyses of [^{125}I]EPO binding (Figure 2) indicated that both CTLL-2-derived (C/EPOR 12B3) and BAF-B03-derived (B/EPOR 12B4) EPOR virus infected clones expressed low-affinity EPOR: expressing 450 and 540 binding sites per cell with K_d values of 545 and 490 pM, respectively. These values were comparable with those detected in Friend virus-induced mouse erythroleukemia cell lines such as K-1 and T3C1-2-0 (Todokoro *et al.*, 1988a). Similar results were obtained with other EPOR virus infected cell clones, C/EPOR 12B6 (CTLL-2-derived) and B/EPOR 3A4 (BAF-B03-derived) (data not shown).

Thus, expression of EPOR with comparable ligand binding affinities can be induced, at almost equal levels to expression in EPOR positive cells, both in the IL-2-dependent CTLL-2 cells and the IL-3-dependent BAF-B03 cells following EPOR virus infection.

Growth characteristics of CTLL-2 cells expressing EPOR

We then examined the growth characteristics of CTLL-2 clones expressing EPOR (C/EPOR 12B3, C/EPOR 12B6).

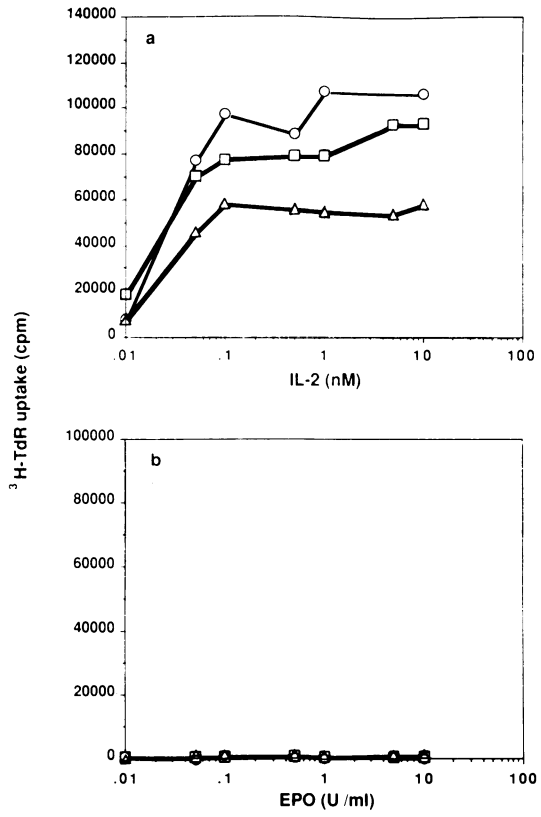

Fig. 3. Growth response of CTLL-2 cells expressing EPOR to IL-2 and EPO. CTLL-2-derived clones expressing EPOR (C/EPOR 12B3 and C/EPOR 12B6) and parental line CTLL-2 were cultured for 3 days with medium containing various concentrations of human IL-2 (**a**) or human EPO (**b**), and the incorporation of [^{3}H]thymidine into the cells was analyzed. ○, CTLL-2; □, C/EPOR 12B3; △, C/EPOR 12B6.

Their growth response to IL-2 and EPO was estimated by measuring [^{3}H]thymidine incorporation into the cells. Both C/EPOR 12B3 and C/EPOR 12B6 responded to IL-2 in a concentration-dependent manner, as did their parental cell line, CTLL-2 (Figure 3a). In contrast, they all did not respond to EPO even at extremely high concentrations (Figure 3b). BAF-B03-derived clones expressing EPOR (B/EPOR 3A4 and B/EPOR 12B4) responded to both IL-3 and EPO (Figure 4) as previously reported (Li *et al.*, 1990; Mori *et al.*, 1990; Yoshimura *et al.*, 1990). Thus, even though EPOR expressed in CTLL-2 cells shows favorable binding activity to EPO it fails to transmit the growth signal in this cell line. The low receptor number (450 binding sites per cell) may not account for this failure, because cell surface expression level of wild type EPOR is also very low (100−1100 sites per cell) in IL-3-dependent transfectants including B/EPOR 3A4 and B/EPOR 12B4, described here, and in erythroid cell lines (Todokoro *et al.*, 1988a; Yoshimura *et al.*, 1990; Carroll *et al.*, 1991; D'Andrea *et al.*, 1991; Miura *et al.*, 1991).

EPOR expressed in CTLL-2 cells mediates EPO internalization without growth signal transduction

In the case of IL-2R, IL-2 is internalized only when it binds to IL-2Rβ which can transduce IL-2 signals to the cells (Fujii *et al.*, 1986; Weissman *et al.*, 1986; Robb and Greene, 1987; Hatakeyama *et al.*, 1989a). We therefore addressed the question of whether the non-responsiveness of C/EPOR 12B3

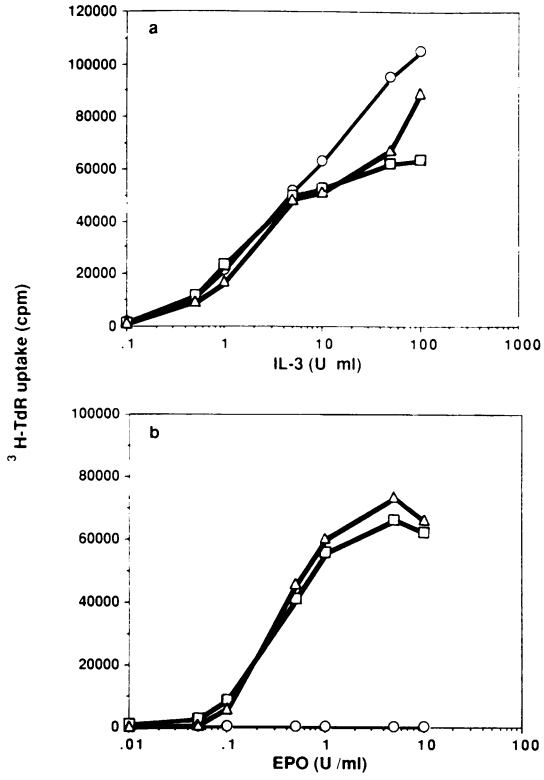

Fig. 4. Growth response of BAF-B03 cells expressing EPO to IL-3 and EPO. BAF-B03-derived clones expressing EPOR (B/EPOR 3A4 and B/EPOR 12B4) and parental line BAF-B03 were cultured for 3 days with medium containing various concentrations of mouse IL-3 (**a**) or human EPO (**b**), and the incorporation of [^{3}H]thymidine into the cells was analyzed. ○, BAF-B03; □, B/EPOR 3A4; △, B/EPOR 12B4.

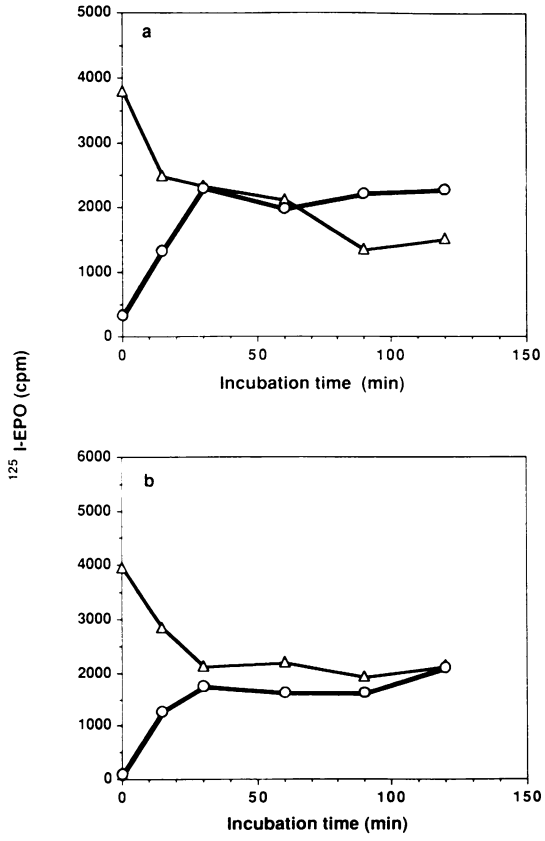

Fig. 5. EPO internalization in IL-2-dependent CTLL-2 clone expressing EPOR (C/EPOR 12B3) and IL-3-dependent BAF-B03 clone expressing EPOR (B/EPOR 12B4). (**a**) C/EPOR 12B3 and (**b**) B/EPOR 12B4. EPOR-mediated internalization of [^{125}I]EPO was examined as described in Materials and methods. At the indicated times the levels of the acid-sensitive (membrane-bound) radioactivity (△) and the acid-resistant (internalized) radioactivity (○) are shown.

cells to EPO is due to their inability to internalize EPO after receptor binding. As shown in Figure 5, [^{125}I]EPO was rapidly internalized in C/EPOR 12B3 cells after binding to EPOR. The internalization kinetics were similar to those of B/EPOR 12B4 cells whose growth was supported by EPO. These results indicate that the non-responsiveness of C/EPOR 12B3 to EPO is not due to a disturbed or decreased internalization of EPO.

Additional expression of the IL-3Rβ subunit AIC2B still does not induce a growth response to EPO in CTLL-2 cells expressing EPOR

Recently it has been shown that one of the mouse IL-3Rβ subunits AIC2B is a common β subunit (signal transducer) of the high-affinity receptors for IL-3, IL-5 and GM-CSF (Devos *et al.*, 1991; Kitamura *et al.*, 1991a; Takaki *et al.*, 1991; Hara and Miyajima, 1992). In fact, coexpression of mouse IL-3Rα or human GM-CSFRα with AIC2B made CTLL-2 cells responsive to the respective ligand. In Figures 3 and 4, we have shown that EPOR was able to transmit its growth signal in IL-3-dependent BAF-B03 cells (constitutively expressing AIC2B) but not in IL-2-dependent CTLL-2 cells (expressing no AIC2B). In addition, when we expressed EPOR in a mouse IL-5-dependent pre-B cell line, Y16 (Tominaga *et al.*, 1989), (constitutively expressing AIC2B), we found that the Y16 clones expressing EPOR (Y/EPOR1, Y/EPOR2) responded to EPO in a concentration-dependent manner (Figure 6). Since there is a good correlation between the competence of EPOR signaling and the expression of endogenous AIC2B in these tested cell lines, we hypothesized that AIC2B may be a common signal transducer for IL-3, IL-5 and EPO. To test this hypothesis, we introduced an AIC2B cDNA into the CTLL-2-derived, EPOR-expressing cell lines. We cotransfected the pME522 plasmid containing a mouse AIC2B cDNA (Gorman *et al.*, 1990) and the pY3 plasmid containing the hygromycin B resistance gene (Blochlinger and Diggelmann, 1984) by electroporation into C/EPOR 12B3 cells. The hygromycin B resistant transfectant clones thus obtained were found to express a reasonably high level of AIC2B as analyzed by flow cytometry using a mouse IL-3Rβ subunit-specific monoclonal antibody, anti-Aic2 (Yonehara *et al.*, 1990) (data not shown). Subsequently, these CTLL-2-derived clones expressing both EPOR and AIC2B (C/EB1 and C/EB2) were examined for their growth characteristics. As shown in Figure 7, neither C/EB1 nor C/EB2 cells exhibited any response to EPO and still required IL-2 for their growth. We also introduced a gene for another β subunit of IL-3R, AIC2A (Itoh *et al.*, 1990), which is considered to be an IL-3-specific subunit, into C/EPOR 12B3 cells, and found that the cells now expressing both EPOR and AIC2A failed to respond to EPO (data not shown). Thus, the lack of AIC2B and AIC2A expression cannot account for the inability of the CTLL-2 cell line to transduce signals from EPOR.

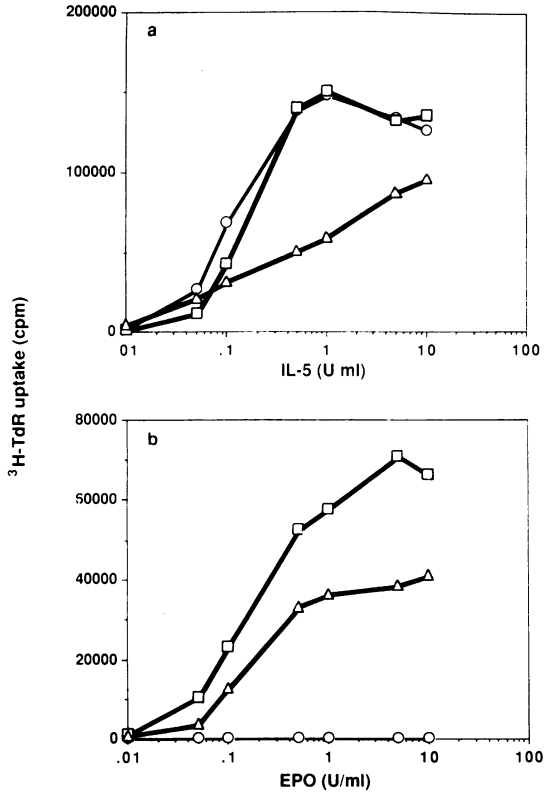

Fig. 6. Growth response of Y16 cells expressing EPOR to IL-5 and EPO. Y16-derived clones expressing EPOR (Y/EPOR1 and Y/EPOR2) and parental line Y16 were cultured for 3 days with medium containing various concentrations of mouse IL-5 (**a**) or human EPO (**b**), and the incorporation of [^{3}H]thymidine into the cells was analyzed. ○, Y16; □, Y/EPOR1; △, Y/EPOR2.

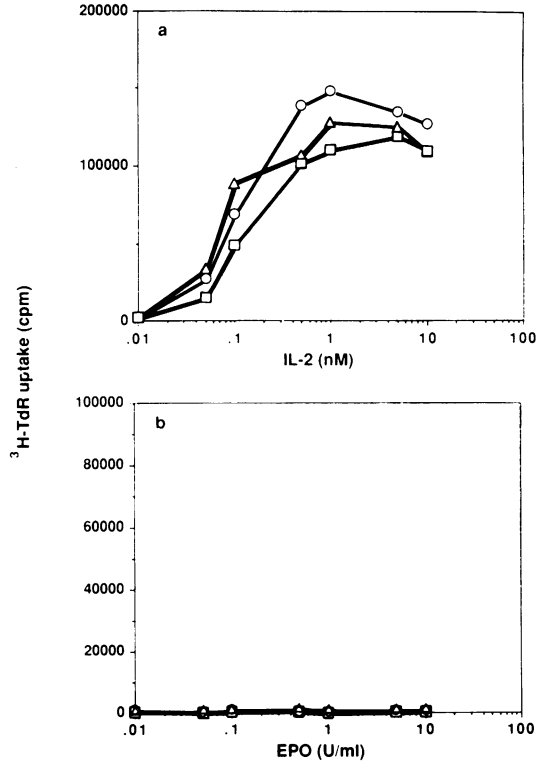

Fig. 7. Growth response of CTLL-2 cells expressing both EPOR and IL-3R β subunit, AIC2B, to IL-2 and EPO. CTLL-2-derived clones expressing both EPOR and AIC2B (C/EB1 and C/EB2) and CTLL-2 clone expressing EPOR (C/EPOR 12B3) were cultured for 3 days with medium containing various concentrations of human IL-2 (**a**) or human EPO (**b**), and the incorporation of [^{3}H]thymidine into the cells was analyzed. ○, C/EPOR 12B3; □, C/EB1; △, C/EB2.

Discussion

Nicola and Metcalf (1991) have noted that the functions of cytokines show redundancy and pleiotropy: several cytokines regulate cells of a particular cell lineage and a single cytokine controls cells of multiple lineages and Arai *et al.* (1990) have proposed a cytokine network and cross-talk among the receptors. Members of the cytokine receptor superfamily can be categorized into several groups based on their structural similarities; for example, (i) EPOR, IL-2Rβ, IL-3Rβ and IL-4R (D'Andrea *et al.*, 1990), (ii) EPOR and IL-2Rβ (D'Andrea *et al.*, 1989b; Cosman *et al.*, 1990), (iii) G-CSFR and IL-4R (Fukunaga *et al.*, 1990), and (iv) G-CSFR and IL-6R gp130 (Hibi *et al.*, 1990; Fukunaga *et al.*, 1991). The members of each subgroup share sequence homologies in their cytoplasmic regions, but not with the members of other subgroups. Also, there are some reports which support cross-talk between the members of each subgroup (Hatakeyama *et al.*, 1989b; Kono *et al.*, 1990; Li *et al.*, 1990; Yoshimura *et al.*, 1990; D'Andrea *et al.*, 1991). These findings suggest that there may be a common downstream signaling pathway for each receptor subgroup, which may enable cross-talk with other members of the same subgroup. Longmore and Lodish (1991) have recently reported that the expression of an activated mutant EPOR, cEpoR, made the IL-2-dependent CTLL-2 cells IL-2 independent. This finding supports the view that EPOR and IL-2R share a common signaling pathway (D'Andrea *et al.*, 1989b, 1991; Kono *et al.*, 1990). Our present study, however, contradicts this hypothesis because the expression of EPOR in IL-2-dependent CTLL-2 cells failed to make the cells EPO dependent. Conversely EPOR expressed in IL-3-dependent BAF-B03 cells and IL-5-dependent Y16 cells did make the cells EPO dependent. EPOR expressed in CTLL-2 cells was able to bind to and internalize EPO, and furthermore, the expression of EPOR in the CTLL-2-derived and BAF-B03-derived cells was almost equal (Figures 2 and 5) and comparable with that seen in erythroleukemia cell lines. We can therefore exclude the possibility that defects in, or a low expression of the EPOR in CTLL-2 cells cause the unresponsiveness of these cells to EPO. From these observations we conclude that the downstream signaling pathways between EPOR and IL-2R are, at least, partially different from each other. Similar findings have been obtained with IL-6 and G-CSF which also could not support the growth of CTLL-2 cells expressing IL-6R and G-CSFR respectively (Fukunaga *et al.*, 1991; Murakami *et al.*, 1991). Accordingly, mere sequence similarities in the cytoplasmic region do not necessarily reflect the existence of common downstream signaling pathways.

Cytokine receptors can also be divided into multiple subgroups based on their usage of one or more common subunits. For instance, the high-affinity receptors for IL-3, IL-5 and GM-CSF share a common β subunit, AIC2B in mice and KH97 in human, which is essential for their signal transduction (Devos *et al.*, 1991; Kitamura *et al.*, 1991a,b; Takaki *et al.*, 1991). A similar receptor subgroup including IL-6R, LIF-R and Oncostatin M receptor (OSM-R) has recently been noted (Gearing *et al.*, 1992). IL-6R gp130, a signal transducer of the IL-6R complex, was also found

to be an affinity converter for LIF-R and OSM-R. Thus, several different cytokine binding subunits may interact with a common second subunit which operates a certain intracellular signaling pathway and elicits specific biological functions. These examples provide evidence for cross-talk among cytokine receptors especially at their subunit level. In the present study, we have examined whether EPOR may share a common subunit with IL-3R, IL-5R and GM-CSFR, the rationale being the finding that exogenously introduced EPOR could transmit growth signals in two AIC2B-expressing cell lines, BAF-B03 and Y16, but could not in an AIC2B-negative cell line, CTLL-2. However, additional expression of AIC2B by itself failed to support EPOR-mediated growth signal transduction in CTLL-2 cells which did express EPOR, although AIC2B molecules here were without upstream stimulation by ligand−receptor binding.

The EPOR cDNA used in this study encodes a protein that constitutes the low-affinity receptor and is believed to associate with a second subunit (D'Andrea *et al.*, 1989a), as yet uncharacterized. Yoshimura and Lodish (1992) have recently reported that EPOR was associated with a putative second subunit, 130 kDa phosphoprotein (pp130), present in the EPOR-expressing Ba/F3 cell line and the EPO-dependent murine erythroleukemia cell line, HCD-57. IL-3 also mediated the phosphorylation of this protein (A.Yoshimura, personal communication). Thus, we cannot currently distinguish the following three possibilities: (i) a second subunit which is a signal transducer of EPOR is also present in the EPOR-expressing CTLL-2 cells but one or more downstream components are missing, (ii) a second subunit for EPOR is missing in CTLL-2 cells and so the downstream signaling of EPOR is not transduced, and (iii) the low-affinity EPOR by itself is sufficient to transduce EPO signal into cells without a second subunit and one or more downstream components are missing in CTLL-2 cells.

The following four points may also be raised in the comparison of signal transduction pathways between EPOR and IL-2R. First, neither IL-4R, IL-6R gp130 nor G-CSFR can transduce their signals from their respective ligand−receptor complexes in CTLL-2 cells although IL-4R is sufficient to transmit the signal from IL-4 without an additional subunit (Mosley *et al.*, 1989). IL-6R gp130 is a signal transducer of IL-6R, and a homodimer of G-CSFR is suggested to bind G-CSF and transduce its signals (Fukunaga *et al.*, 1991; Murakami *et al.*, 1991). These results suggest that these receptors may utilize a certain downstream pathway distinct from that of IL-2R. Since EPOR may undergo dimerization (D'Andrea *et al.*, 1989a; Watowich *et al.*, 1992), it is likely that EPOR exists as a homodimer which is sufficient to transduce signals into cells of its natural target cell lineage, as is the case for G-CSFR but one or more downstream target molecules may be missing in CTLL-2 cells.

Secondly, chimeric receptors containing the extracellular region of murine EPOR and the cytoplasmic region of another murine IL-3R β subunit, AIC2A, can transduce EPO signal in IL-3-dependent Ba/F3 cells (Zon *et al.*, 1992), although chimeric receptors containing the extracellular region of IL-2Rβ and the cytoplasmic region of EPOR cannot transduce IL-2 signals in IL-3-dependent BAF-B03 cells (Mori *et al.*, 1990). In addition, we and others have observed that exogenously introduced EPOR transduces EPO signal in Ba/F3 and BAF-B03 cells (Li *et al.*, 1990; Mori *et al.*, 1990; Yoshimura *et al.*, 1990). These results indicate that an EPO signal is transduced through the AIC2A's cytoplasmic region, but an IL-2 signal is not transduced through the EPOR's cytoplasmic region. However, the EPOR−AIC2A chimeric receptors can also transduce EPO signal in IL-2-dependent CTLL-2 cells (Zon *et al.*, 1992), in contrast to our present results which indicate that EPOR coexpressed with AIC2A cannot transduce EPO signal in CTLL-2 cells. These observations suggest that the downstream signaling pathways may be similar between EPOR and IL-3R and between IL-2R and IL-3R, but may be, at least, partially different between EPOR and IL-2R. Takeshita *et al.* (1992) recently isolated a cDNA clone for the third molecule of IL-2R complex (IL-2Rγ), which binds to IL-2Rβ to form high- and intermediate-affinity IL-2R complexes, enabling internalization of those complexes. EPOR as characterized in murine erythroleukemia cells, is known to provide only a low-affinity moiety. However, EPOR molecules in EPOR-introduced COS-1 cells (D'Andrea *et al.*, 1989) and in an erythroleukemia cell line, SKT6, which is differentiation-inducible by EPO (Todokoro *et al.*, 1987) have high affinity. It is still questionable whether there are second and third molecules corresponding to IL-2R α and γ in the EPO−EPOR signaling system, especially in erythroid precursor cells.

Thirdly, the C-terminal residues of the cytoplasmic domain of exogenously introduced EPOR have been shown to down-modulate the responsiveness of myeloid FDC-P1 cells to GM-CSF (Quelle and Wojchowski, 1991b). Since exogenously introduced EPOR transmits an EPO signal in FDC-P1 cells, it has been suggested that EPOR and GM-CSFR may share common target molecules in their growth signaling and may compete with each other in binding with these target molecules. Alternatively, EPOR may interact with GM-CSFR and inhibit the formation of the high-affinity GM-CSFR which transduces GM-CSF signals. In the present study, however, we showed that the β subunit of GM-CSFR, AIC2B, by itself did not participate in EPOR-mediated growth signal transduction in EPOR-expressing CTLL-2 cells. It is thus unlikely that EPOR competes with GM-CSFR to bind to AIC2B to inhibit the formation of high-affinity GM-CSFR although this is not formally excluded. EPOR may possibly interact with the downstream target molecules of some cytokine receptors and thereby modulate the functions of those receptors, but may not interact with other types of cytokine receptors such as IL-2R reflecting the different biological functions and the different target cell lineages.

Fourthly, Shibuya *et al.* (1992) have shown that IL-2R β mediates at least two signaling pathways. The first pathway may be regulated by protein tyrosine kinases of the src family such as $p56^{lck}$ and $p59^{lyn}$ and may lead to expression of c-Fos and c-Jun. The second pathway leads to c-*myc* gene induction. IL-3 stimulation of IL-3R in BAF-B03 cells can activate both pathways but EGF stimulation of EGFR in BAF-B03 cells, expressing exogenously introduced EGFR, activates only the first pathway. Since EPO has been reported to increase c-*myc* mRNA levels (Todokoro *et al.*, 1988b), EPO-stimulated EPOR may activate the second pathway in BAF-B03 cells but neither pathway in CTLL-2 cells. Although EPOR may not be associated with $p56^{lck}$ and $p59^{lyn}$, it may have other target molecules that mediate signals to c-*myc*. There may be other intervening tyrosine kinases as is the case for activation of interferon responsive genes via interferon α/β receptors (Fu, 1992; Velazquez *et al.*, 1992).

Further studies are necessary to clarify the difference between EPOR and IL-2R in their downstream signaling pathways especially in the natural target cells of EPO and IL-2 *in vivo*. However, on the basis of our present data as well as the findings discussed above, we may conclude at this moment that the EPOR signaling pathways are at least partially different from the IL-2R signaling pathways, although EPOR and IL-2R may share some distant target molecules such as c-*myc*. Previous studies have also demonstrated that EPO rapidly induces phosphorylation of cellular proteins, including EPOR, at their tyrosine residues in mouse cell lines (Quelle and Wojchowski, 1991a) and that EPO induces the rapid phosphorylation of Raf-1 at both serine and tyrosine residues and induces activation of Raf-1 kinase (Carroll *et al.*, 1991). IL-3 also induces the phosphorylation of the same proteins, with the exception of EPOR, suggesting that the mediators for downstream signaling of EPOR and IL-3R may, at least, partially overlap.

The IL-2- and IL-5-dependent cell lines expressing EPOR which we describe in this report will be valuable for studying similarities and differences between these structurally related, but physiologically distinct cytokine receptors in their downstream signaling mechanisms. Accordingly, we are now attempting to find the molecules which may rescue EPO-mediated growth signal transduction in EPOR-introduced CTLL-2 cells. This kind of study may clarify the complex signaling networks for the cell growth by applying such modern technologies as differential cloning and complementation cloning.

Materials and methods

Cells and cell culture

The IL-2-dependent mouse cytotoxic T cell line, CTLL-2 (Cerottini *et al.*, 1974) was maintained in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS) (Biocell, Carson, CA) and 1 nM recombinant human IL-2 which was the gift of Dr J.Hamuro (Ajinomoto Co., Tokyo). The BAF-B03 cell line (Hatakeyama *et al.*, 1989b), a subline of an IL-3-dependent mouse pro-B cell line Ba/F3, was the gift of Dr T.Taniguchi (Osaka University, Osaka). BAF-B03 cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) FBS and 20% (v/v) conditioned medium from the WEHI-3B cells as a source of IL-3. An IL-5-dependent mouse pre-B cell line, Y16 (Tominaga *et al.*, 1989) was the gift of Dr K.Takatsu (University of Tokyo, Tokyo). Y16 cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) FBS and 5 U/ml of recombinant mouse IL-5 also supplied by Dr K.Takatsu.

Plasmid construction

For construction of pLEPORSN, the 1.5 kb cDNA insert was excised from pMER2-1 (Kuramochi *et al.*, 1990), which was the gift of Dr K.Todokoro (The Institute of Physical and Chemical Research, Tsukuba), by *Sty*I and the fragment was blunt-ended using T4 DNA polymerase. This fragment was redigested with *Eco*RI and then inserted between the *Hpa*I and *Eco*RI sites of a pLXSN retroviral vector (Miller and Rosman, 1989).

Retroviral infection of cells

Retroviral infection was performed as previously described (Li *et al.*, 1990). CTLL-2, BAF-B03 and Y16 cells were infected with retroviral supernatants from PA317 packaging cell clones which were transfected with the EPOR cDNA expression vector, pLEPORSN. Selection was initiated 24 h after infection using 1 mg/ml (for CTLL-2), 2 mg/ml (for BAF-B03) or 500 μg/ml (for Y16) of G418 (GIBCO, Grand Island, NY). Subclones were isolated from pLEPORSN infected cells by limiting dilution.

DNA transfection

Plasmid DNAs were transfected into C/EPOR 12B3 cells by electroporation as previously described (Kitamura *et al.*, 1991a). Briefly, 40 μg of the AIC2B expression plasmid pME522 (Gorman *et al.*, 1990), which was given us by Dr A.Miyajima (DNAX, Palo Alto, CA), was linearized by *Sca*I digestion and cointroduced with 4 μg of *Hin*dIII-digested hygromycin B phosphotransferase expression plasmid pY3 (Blochlinger and Diggelmann, 1984) into 5×10^6 cells by electroporation. Selection was initiated 48 h after transfection in medium containing 1 mg/ml hygromycin B (Sigma, St Louis, MO) and 1 nM IL-2. Subclones of the transfectants were obtained by limiting dilution.

EPO binding and internalization assay

Radiolabeling of recombinant human EPO (the gift of Kirin Brewery Co., Tokyo) and EPO binding assay were carried out as described elsewhere (Sawyer *et al.*, 1987a). Briefly, cells (5×10^6) were incubated with serial dilution of ^{125}I-labeled EPO for 4 h at 10°C in the presence or absence of a 100-fold excess of unlabeled EPO. After incubation, cell-bound and free EPO were separated by centrifugation through an oil cushion. Non-specific binding was estimated by the binding assay in the presence of a 100-fold excess of unlabeled EPO. For the EPO internalization assay, cells (1×10^7) were incubated with 500 pM [^{125}I]EPO for 4 h at 10°C. The cell suspension was then quickly warmed to 37°C and at the indicated times thereafter two aliquots of cells were removed. One aliquot was centrifuged through an oil cushion and the radioactivity of the cell pellet was measured to determine the level of total cell-associated EPO, while the other aliquot was treated with acid buffer (0.5 M NaCl, 0.25 M acetic acid, pH 2.5) to determine the level of internalized EPO. At the same time, non-specific binding was estimated in the presence of 100 nM unlabeled EPO.

Cell proliferation assay

Cells (1×10^3) were cultured with various concentrations of recombinant human EPO, human IL-2, mouse IL-3 (also provided by Dr A.Miyajima) or mouse IL-5 in RPMI-1640 supplemented with 10% FBS in a 96-well microculture plate for 72 h at 37°C. The cells were pulse-labeled with 0.5 μCi of [^{3}H]thymidine for 4 h prior to harvest. The [^{3}H]thymidine incorporation was measured to examine the effects of EPO, IL-2 and IL-3 on the proliferation of cells.

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