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A pathway distinct from the mammalian unfolded protein response regulates expression of endoplasmic reticulum chaperones in non-stressed cells

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The stress-induced unfolded protein response (UPR) is the only signaling pathway known to regulate expression of genes encoding the resident endoplasmic reticulum (ER) molecular chaperones and folding enzymes, yet these genes are constitutively expressed in all cells. We have examined the expression of ER chaperones in several cell lines that are dependent on a variety of cytokines for growth and survival. When the various cell lines were deprived of essential growth factors, mRNA levels of the ER chaperones BiP and GRP94 decreased dramatically. Re-stimulation of ligand-deprived cells with the appropriate growth factor induced *BiP* and *GRP94* as delayed-early response genes. Cytokine induction of BiP and GRP94 biosynthesis was not preceded by a burst of glycoprotein traffic through the ER nor accompanied by expression of the CHOP transcription factor. The glycosylation inhibitor tunicamycin potently induced expression of both ER chaperones and CHOP in ligand-deprived cells, demonstrating that the UPR pathway remains functionally intact in the absence of growth factor-mediated signaling. Therefore, basal expression of ER chaperones is dependent upon and regulated by a mitogenic pathway distinct from the stress-inducible UPR cascade and this probably controls expression of ER chaperones and folding enzymes needed to assist protein biogenesis in the ER of normal, non-stressed cells.

Keywords: endoplasmic reticulum/molecular chaperone/protein folding/unfolded protein response

Introduction

The biogenesis of nascent proteins translocated into the calcium-rich, oxidizing milieu of the endoplasmic reticulum (ER) lumen is assisted by a group of resident ER proteins that include molecular chaperones and folding enzymes. These specialized ER proteins are constitutively expressed in all cells, where they play a role in monitoring and assisting the maturation of normal proteins (Gething and Sambrook, 1992; Hendrick and Hartl, 1993) and are essential for proper steady-state operations of the eukaryotic secretory pathway. Expression of mutant secretory pathway proteins or exposure of cells to agents that adversely affect ER protein folding and maturation all result in the accumulation of unfolded proteins in the ER, thereby activating an inter-organelle signaling pathway linking the ER and nucleus. This response, termed the unfolded protein response (UPR), includes the coordinate transcriptional up-regulation of ER chaperones and folding enzymes (Lee, 1992).

In yeast, an ER transmembrane serine/threonine kinase, Ire1p, apparently serves as a proximal effector of the UPR signaling cascade (Cox *et al.*, 1993; Mori *et al.*, 1993). When unfolded proteins accumulate in the ER, the Ire1p kinase is activated by oligomerization and transphosphorylation in a manner reminiscent of higher eukaryotic growth factor receptors (Shamu and Walter, 1996; Welihinda and Kaufman, 1996). Following Ire1p activation, the Hac1p transcription factor binds to an element termed the UPRE (unfolded protein response element) and activates transcription of UPR-induced genes (Cox and Walter, 1996) such as *KAR2* that encodes the yeast homolog of the mammalian ER chaperone BiP/GRP78. An intact UPR relies on functional Ire1p and Hac1p and is essential for yeast survival under conditions of ER stress (Cox *et al.*, 1993; Mori *et al.*, 1993). Importantly, this pathway is not required for normal growth conditions when ER chaperones and folding enzymes are expressed at basal levels. Basal expression of yeast BiP is regulated by a separate, but poorly defined, GC-rich region in the *KAR2* promoter (Mori *et al.*, 1992; Kohno *et al.*, 1993).

A mammalian homolog of either the Ire1p kinase or Hac1p has not been identified, yet inhibitor studies suggest that both serine/threonine and tyrosine phosphorylation are involved in the UPR (Price and Calderwood, 1992; Cao *et al.*, 1995). The mammalian *BiP* promoter contains a region that shares partial homology with the UPRE and is involved in stress-inducible *BiP* expression (Chang *et al.*, 1989; Li *et al.*, 1994, 1997). This element, termed the GRP core, is also found in the promoters of other mammalian ER chaperone genes such as *GRP94* (Chang *et al.*, 1989). In contrast to the yeast UPRE, the GRP core apparently acts in concert with a variety of other promoter sequences, including a cAMP response element and a series of CAAT and CAAT-like motifs, to activate the *BiP* promoter fully in response to ER stress (Alexandre *et al.*, 1991; Roy and Lee, 1995). Current information regarding the *BiP* promoter reveals no distinction between elements controlling basal versus stress-inducible *BiP* expression (Lee, 1992).

The mammalian UPR also includes induction of *CHOP* (C/EBP homologous protein; also known as *GADD153*, growth arrest and DNA damage), a member of the C/EBP family of transcription factors (Carlson *et al.*, 1993; Wang *et al.*, 1996). *CHOP/GADD153* was identified originally in a screen designed to isolate genes induced by DNA damage (Fornace *et al.*, 1988), and may actually be a

target of multiple inducible pathways such as the cellular response to UV-mediated DNA damage (Luethy and Holbrook, 1992; Gujuluva *et al.*, 1994) and the lipopolysaccharide (LPS)-induced acute phase response in liver cells (Sylvester *et al.*, 1994). Additionally, a large body of evidence indicates that induction of *CHOP* expression represents a hallmark of the mammalian ER stress response. First, *CHOP*, like the genes encoding ER chaperones and folding enzymes, is induced by agents that specifically interfere with protein folding in the ER (Bartlett *et al.*, 1992; Chen *et al.*, 1992; Price and Calderwood, 1992; Carlson *et al.*, 1993). In fact, all stimuli known to up-regulate ER chaperone expression have been found to induce *CHOP* coordinately with similar kinetics and dose responsiveness (Halleck *et al.*, 1997). Second, the induction of *CHOP* in response to ER stress-inducing agents, such as the glycosylation inhibitor tunicamycin, is markedly attenuated in CHO cells stably overexpressing an exogenously supplied *BiP* cDNA (Wang *et al.*, 1996). These transfectants are also blocked in their ability to up-regulate expression of endogenous *BiP* and other ER chaperones during conditions of ER stress (Dorner *et al.*, 1992). Therefore, induction of both the ER chaperone genes and *CHOP* in response to agents that adversely affect protein folding in the ER is dependent upon a BiP-mediated signal originating in the stressed ER.

A function for CHOP in the UPR has not been delineated. However, CHOP has been implicated in cellular growth arrest (Barone *et al.*, 1994), and unlike the ER chaperone genes induced by the UPR, *CHOP* is not constitutively expressed under normal growth conditions (Ron and Habener, 1992; Wang *et al.*, 1996). This distinction, coupled with the fact that the yeast UPR is dispensable under normal conditions, suggests that basal and stress-inducible expression of the ER chaperones and folding enzymes are controlled by separate pathways. Here, we report that growth factors positively regulate expression of the ER chaperones by a pathway distinct from the stress-induced UPR cascade. We propose that basal expression of critical ER chaperones is mediated by extracellular stimuli (e.g. growth factors) and that cells have the capacity to modulate expression of ER chaperones in response to growth and/or differentiation signals, as well as to signals emanating from the stressed ER.

Results

Expression of ER chaperones is growth factor dependent

As a first step in characterizing the mechanism(s) that might control expression of the ER chaperones under conditions not associated with ER stress, we compared steady-state mRNA levels of *BiP* and *GRP94* in asynchronously growing cells with those in deprived of cytokines required for proliferation and survival. In a variety of cell lines from different lineages and dependent on various growth factors, levels of *BiP* and *GRP94* mRNA dramatically decreased following growth factor deprivation (see Table I).

The rate of protein synthesis declines in cells deprived of essential growth factors. We therefore determined whether the decrease in *BiP* and *GRP94* transcripts in factor-depleted cells reflected a negative feedback mechanism operative during conditions of diminished protein traffic through the ER. We examined the steady-state level of *BiP* and *GRP94* transcripts in interleukin (IL)-3 dependent FDC-P1.2 myeloid cells that were either treated with the protein synthesis inhibitor cycloheximide or shifted to medium lacking IL-3. Greater than 95% of total protein synthesis is blocked in FDC-P1.2 cells within minutes of cycloheximide exposure, while cells deprived of IL-3 for 6 h maintain protein synthesis, including glycoprotein synthesis, at a level that is ~50% of normal (data not shown). Northern hybridization analyses (Figure 1) revealed that depriving the cells of IL-3 for only 6 h resulted in a substantial decrease of both *BiP* and *GRP94* mRNAs (6 h, 40% decrease; 14 h, 56% decrease). By contrast, the mRNA level for *OST48*, the 48 kDa subunit

Table I. ER chaperone expression is growth factor dependent in multiple cell lineages

Cell line	Lineage	Required growth factor	% mRNA after factor starvation	
			BiP	*GRP94*
32D.3	myeloid	IL-3	24	18
FDC-P1.2	myeloid	IL-3	27	27
DA-3	myeloid	IL-3	26	30
FL5.12	pro-B	IL-3	13	15
Bac1.2F5	macrophage	CSF-1	37	39

Cells were deprived of their required growth factor for the indicated periods and mRNA levels were quantitated by PhosphorImager analysis: 32D.3, 15 h; FDC-P1.2, 20 h; DA-3, 24 h; FL5.12, 15 h; Bac1.2F5, 18 h.

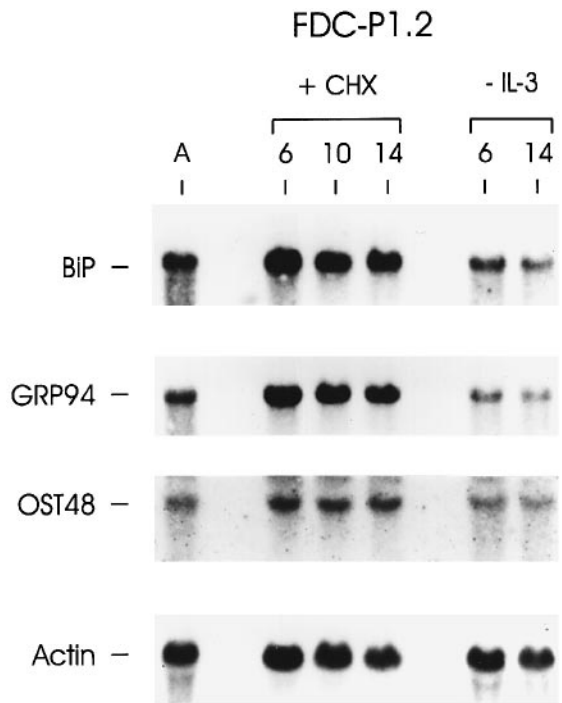

Fig. 1. Expression of ER chaperones is growth factor dependent. IL-3-dependent myeloid FDC-P1.2 cells asynchronously growing in IL-3 (A) were either treated with cycloheximide (CHX) to block protein synthesis or deprived of IL-3 for the indicated number of hours. Cells were >95% viable after 14 h of either cycloheximide exposure or IL-3 starvation. Expression of *BiP*, *GRP94*, *OST48* and *actin* was assessed by Northern blotting.

of the mammalian oligosaccharyl tranferase complex (Silberstein *et al.*, 1992), was less affected by IL-3 withdrawal (Figure 1; 6 h, 23% decrease; 14 h, 31% decrease), indicating that growth factor deprivation does not lead to an equivalent overall decrease in the expression of genes encoding ER components. Unlike the effect of growth factor withdrawal, cessation of protein synthesis by cycloheximide inhibition, even for 14 h, did not diminish levels of *BiP* and *GRP94* transcripts. Rather, cycloheximide mildly induced (~2-fold) both *BiP* and *GRP94* mRNA levels. Therefore, the diminishing expression of *BiP* and *GRP94* in growth factor-depleted cells cannot be attributed simply to a reduced need for ER chaperones when protein traffic through the ER declines.

In many cell systems, removal of the required growth factor leads to growth arrest followed by apoptosis (Metcalf, 1989). We therefore tested whether induced apoptosis or cell cycle arrest were responsible for the decrease in *BiP* and *GRP94* expression following withdrawal of essential growth factors. c-Myc functions as a master regulator of the cell cycle, and its overexpression in IL-3-dependent myeloid 32D.3 cells leads to both continuous cell cycle progression and accelerated apoptosis in the absence of IL-3 (Askew *et al.*, 1991). However, *BiP* and *GRP94* mRNA levels were still down-regulated following IL-3 withdrawal in c-*myc*-over-expressing cells relative to neo controls (Figure 2A). Enforced expression of the cell death antagonist Bcl-2 protects cells from many inducers of death (Yang and Korsmeyer, 1996), and its overexpression in 32D.3 cells markedly suppresses apoptosis following IL-3 withdrawal, with cells surviving in a G_0/G_1 state (data not shown). However, levels of *BiP* and *GRP94* transcripts were down-regulated in Bcl-2-overexpressing cells in a fashion similar to neo controls (Figure 2B). Four separate clones over-expressing Bcl-2 were examined, and each exhibited the same kinetics and magnitude of *BiP* and *GRP94* mRNA decrease in the absence of IL-3 (data not shown). Similar results were obtained in IL-3-dependent pro-B FL5.12 cells overexpressing Bcl-2 or the Bcl-2-related cell death antagonist, Bcl-X_L (Boise *et al.*, 1993) (data not shown). Thus, the down-regulation of ER chaperone expression during growth factor deprivation is not related to events controlling cell cycle progression or apoptosis.

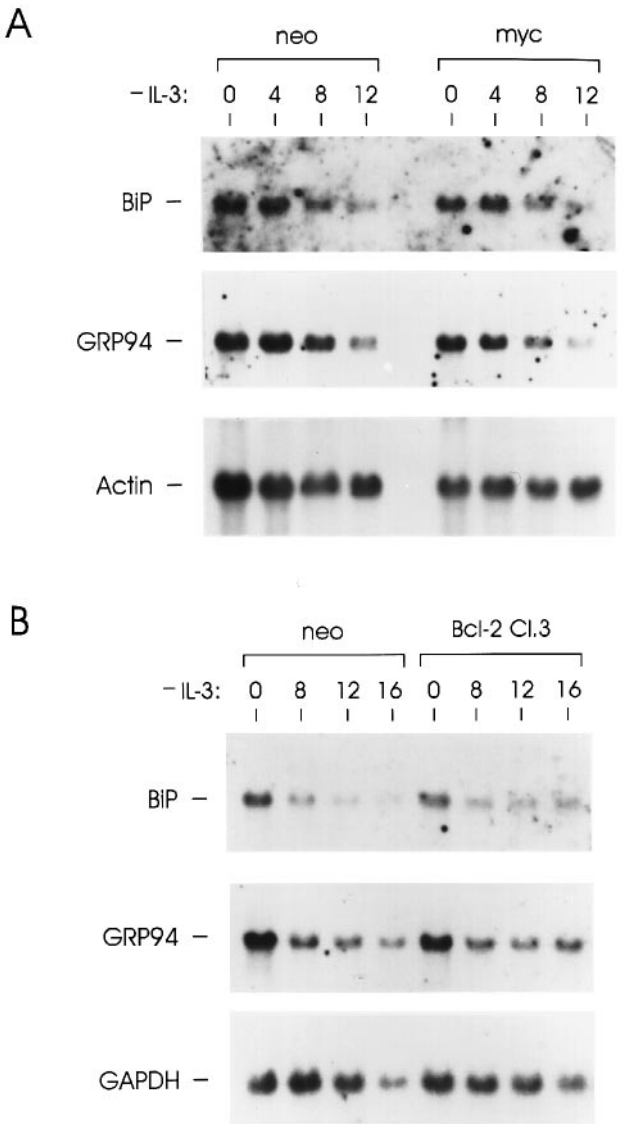

Fig. 2. Growth factor regulation of ER chaperone expression is independent of events controlling cell cycle progression and apoptosis. IL-3-dependent myeloid 32D.3 cells were deprived of IL-3 for the indicated number of hours. Expression of *BiP*, *GRP94*, *actin* and *GAPDH* was assessed by Northern blotting. (**A**) Analysis of pooled 32D.3 transfectants stably overexpressing c-*myc* (myc) and control transfectants expressing the neomycin resistance gene (neo). 32D.3/*c-myc* transfectants exhibited a normal distribution (40–50%) of cells in S phase after 12 h of IL-3 starvation (Askew *et al.*, 1991). (**B**) Analysis of a 32D.3 clone stably overexpressing Bcl-2 and a control clone expressing the neomycin resistance gene. 32D.3/Bcl-2 cells were >90% viable for 48 h in the absence of IL-3.

Growth factors induce expression of ER chaperone genes

To test the hypothesis that basal transcription of ER chaperone genes might be controlled by growth factors, we deprived 32D.3 myeloid cells of IL-3 for 13 h and then re-stimulated the cells with IL-3. Levels of *BiP* and *GRP94* mRNA were examined at various times after growth factor addition. Northern hybridization analyses (Figure 3) revealed that both *BiP* and *GRP94* mRNA decreased as expected during IL-3 starvation and were then induced by re-stimulation with IL-3. The induction occurred with delayed response kinetics (within 2–4 h) and restored *BiP* and *GRP94* mRNA to levels equivalent to or slightly greater than those observed in asynchronously growing cells. By contrast, induction of the early response gene c-*myc* could be seen as early as 30 min after IL-3 re-addition and was fully induced by 2 h. As expected, the expression of both Max, the partner of c-Myc, and Fli-1, an Ets family transcription factor, were relatively unaffected by IL-3 withdrawal or stimulation, and thus served as RNA loading controls. Interestingly, the transcription factor CHOP, a C/EBP family member coordinately induced with ER chaperones in response to ER stress, was not induced by either IL-3 starvation or ligand stimulation of 32D.3 cells. Similar results concerning CHOP were obtained with IL-3-dependent myeloid FDC-P1.2 cells (see below, Figures 4 and 9) and pro-B FL5.12 cells (data not shown). Therefore, the induction of ER chaperones by growth factors is by a pathway distinct from those regulating CHOP.

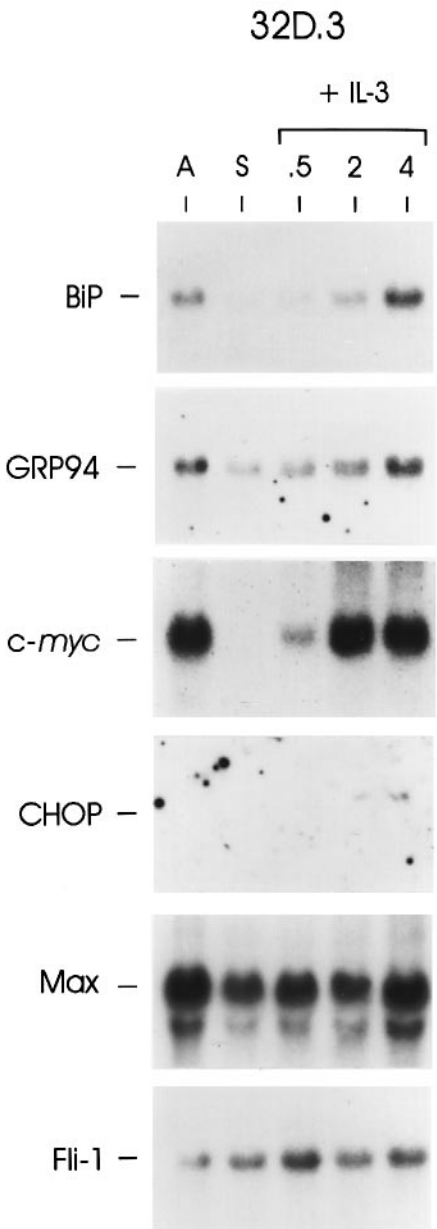

Fig. 3. Growth factors induce expression of ER chaperones with delayed-response kinetics. 32D.3 cells asynchronously growing in IL-3 (A) were starved of IL-3 for 13 h (S) and then re-stimulated with IL-3 for the indicated number of hours. Northern hybridization analyses are shown for *BiP*, *GRP94*, c-*myc*, *CHOP*, *Max* and *Fli-1*.

The delayed kinetics of *BiP* and *GRP94* induction by IL-3 suggested that it might require *de novo* protein synthesis. To address this issue, IL-3-dependent FDC-P1.2 myeloid cells were deprived of IL-3 and then re-stimulated with IL-3 in the presence or absence of cycloheximide. As expected, FDC-P1.2 cells deprived of IL-3 exhibited a marked decrease in *BiP* and *GRP94* mRNA levels and, upon re-stimulation with IL-3, both *BiP* and *GRP94* were potently induced with kinetics similar to those observed in 32D.3 cells (Figure 4). Cycloheximide treatment alone did not influence *BiP* and *GRP94* mRNA levels in factor-depleted cells, but completely blocked the ability of IL-3 to induce *BiP* and *GRP94* expression. Consistent with results from 32D.3 cells (Figure 3), IL-3 re-addition did not coordinately induce *CHOP* expression with the ER

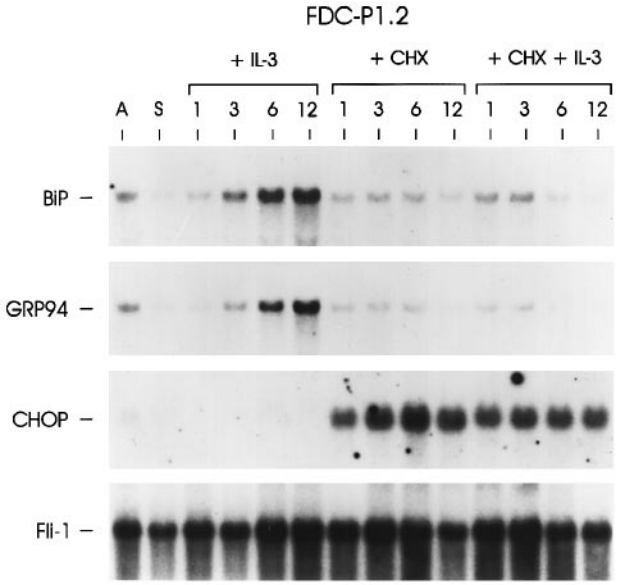

Fig. 4. Induction of ER chaperone gene expression by growth factors requires *de novo* protein synthesis. IL-3-dependent myeloid FDC-P1.2 cells asynchronously growing in IL-3 (A) were starved of IL-3 for 20 h (S) and then either re-stimulated with IL-3 (+ IL-3), treated with the protein synthesis inhibitor cycloheximide (+ CHX) or re-stimulated with IL-3 in the presence of cycloheximide (CHX + IL-3) for the indicated number of hours. Northern hybridization analyses are shown for *BiP*, *GRP94*, *CHOP* and *Fli-1*.

chaperones in FDC-P1.2 cells (Figure 4). *CHOP* can be potently induced by cycloheximide (unpublished data); thus, its induction in IL-3-deprived cells treated with cycloheximide was not surprising (Figure 4) and provided a positive control for *CHOP* induction in these cell lines. Similar experiments in 32D.3 myeloid cells and FL5.12 pro-B cells generalized these findings and demonstrated that new protein synthesis is required for optimal induction of ER chaperone genes by IL-3, characterizing these as delayed-early response genes.

Induction of ER chaperone expression by growth factors requires a mitogenic signal

To characterize the mechanism by which growth factors stimulate expression of ER chaperones, we determined whether growth factors must signal through a mitogenically active receptor to activate their expression. Erythropoietin (Epo) signals through a single chain receptor (EpoR) to induce proliferation and differentiation of erythroid cells (Ihle, 1995). The EpoR has been characterized extensively by mutational analysis and functions as a mitogenically active receptor when expressed in IL-3-dependent myeloid cells (Miura *et al.*, 1993). To determine the requirements of the EpoR for inducing the ER chaperones, we utilized stable transfectants of the IL-3-dependent DA-3 myeloid cell line expressing various forms of the EpoR (Miura *et al.*, 1993). Wild-type EpoR is mitogenically active in DA-3 cells in response to Epo (Miura *et al.*, 1993), activating a Jak–Stat (Jak2–Stat5) pathway via its cytoplasmic membrane-proximal domain (Witthuhn *et al.*, 1993; Ihle, 1995). As shown in Figure 5, the mitogenic wild-type EpoR expressed in DA-3 cells induces *BiP* expression in response to Epo equally as well as the endogenous IL-3 receptor in response to IL-3. A

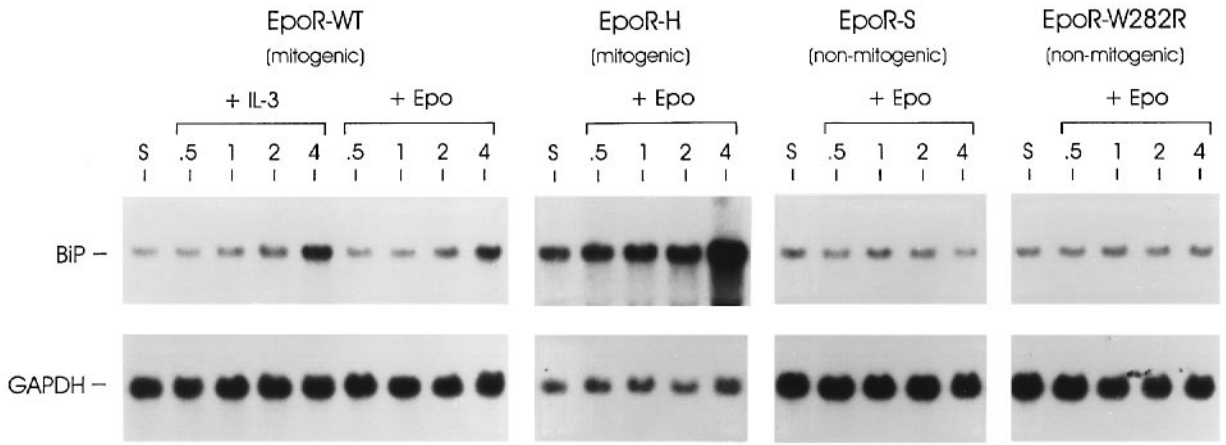

Fig. 5. Induction of ER chaperone gene expression by growth factors requires a mitogenic signal. Transfectants of IL-3-dependent myeloid DA-3 cells stably expressing the wild-type erythropoietin receptor (EpoR-WT), a mitogenically active C-terminal deletion mutant that exhibits enhanced signaling (EpoR-H), a non-mitogenic C-terminal/membrane proximal deletion mutant (EpoR-S) and a non-mitogenic point mutant (EpoR-W282R) were starved of IL-3 for 24 h (S) and then re-stimulated with IL-3 or Epo for the indicated number of hours. Northern hybridization analyses are shown for *BiP* and *GAPDH*. Results similar to those for *BiP* were obtained with the *GRP94* probe (data not shown).

108 amino acid C-terminal deletion mutant of the EpoR (EpoR-H) has been constructed that remains mitogenic, activates the Jak–Stat pathway (Miura *et al.*, 1993; Witthuhn *et al.*, 1993) and exhibits enhanced signaling due to the loss of critical tyrosine residues involved in its negative regulation by the hematopoietic cell phosphatase (HCP) (Yi *et al.*, 1995). Stimulation of cells expressing the mitogenic EpoR-H mutant resulted in an enhanced induction of *BiP* expression, consistent with its increased signaling capacity (Figure 5). Two non-mitogenic EpoR mutants were also examined, EpoR-S and EpoR-W282R. EpoR-S contains a C-terminal 146 amino acid deletion, whereas EpoR-W282R contains a point mutation in the membrane-proximal domain of the EpoR (Miura *et al.*, 1993). Both are unable to activate the Jak pathway and are therefore non-mitogenic receptors (Miura *et al.*, 1993; Witthuhn *et al.*, 1993). EpoR-S and EpoR-W282R both failed to induce *BiP* in response to Epo (Figure 5). Therefore, a mitogenic signal is required for growth factor induction of ER chaperone expression.

Growth factors induce the biosynthesis of ER chaperones

To verify that growth factor regulation of ER chaperones at the mRNA level corresponds to increased biosynthesis of these proteins, we determined the rate of BiP translation in IL-3-starved and stimulated FDC-P1.2 myeloid cells. In parallel, we monitored the synthetic rate of total proteins ([^{35}S]methionine and [^{35}S]cysteine incorporation) and of glycoproteins ([^{3}H]mannose incorporation) that must traffic through the ER. Both total and glycoprotein synthesis were decreased dramatically in FDC-P1.2 cells cultured in the absence of IL-3 and, upon re-stimulation with IL-3, both were restored to near normal levels within 12 h (Figure 6). The rate of translation for BiP paralleled that of total and glycoprotein synthesis, declining ≈90% in factor-depleted cells. However, upon re-stimulation with IL-3, induction of BiP biosynthesis was readily observed within 3 h and continued to rise through 12 h to a point 2.3-fold higher than the basal level (Figure 6). Growth factor stimulation of BiP biosynthesis did not reflect a need to restore a depleted pool of BiP in growth factor-deprived cells. In keeping with the long half-life of BiP (>24 h), the absolute amount of BiP in 32D.3 and

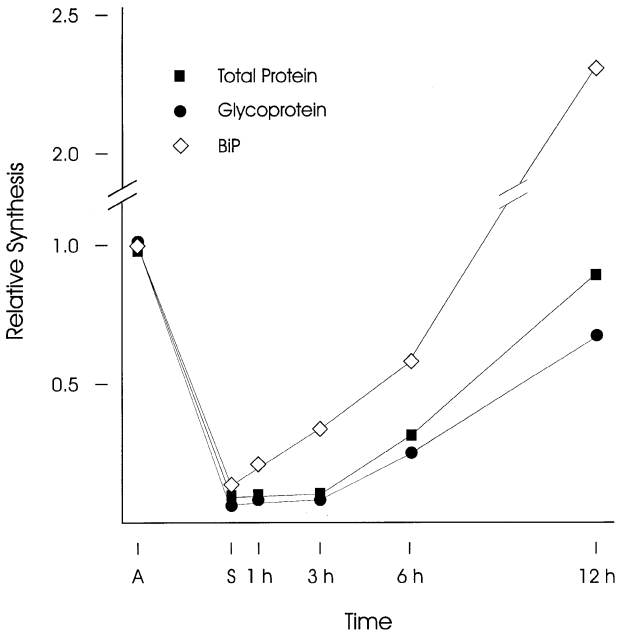

Fig. 6. Induction of BiP biosynthesis in response to IL-3 is not preceded by a burst of glycoprotein synthesis in the ER. Asynchronously growing IL-3-dependent FDC-P1.2 cells (A) were starved of IL-3 for 20 h (S) and then re-stimulated with IL-3 for the indicated number of hours. At each time point, 5×10^6 viable cells were metabolically labeled for 30 min with either [^{35}S]methionine and [^{35}S]cysteine (Total Protein) or [^{3}H]mannose (Glycoprotein). Incorporation of radiolabeled amino acids and mannose was monitored by determining the TCA-precipitable activity in labeled cell lysates. BiP was immunoprecipitated from the [^{35}S]methionine- and [^{35}S]cysteine-labeled cell lysates with a rat monoclonal anti-BiP antibody and resolved by SDS–PAGE under reducing conditions. Labeled BiP was detected and quantitated by PhosphorImager analysis of the dried gel. Relative synthesis of total protein, glycoprotein and BiP was calculated by normalizing the TCA-precipitable activity (both ^{35}S and ^{3}H) in labeled cell lysates and the value for BiP obtained for each time point to those observed in the asynchronous population (A) (set at 1).

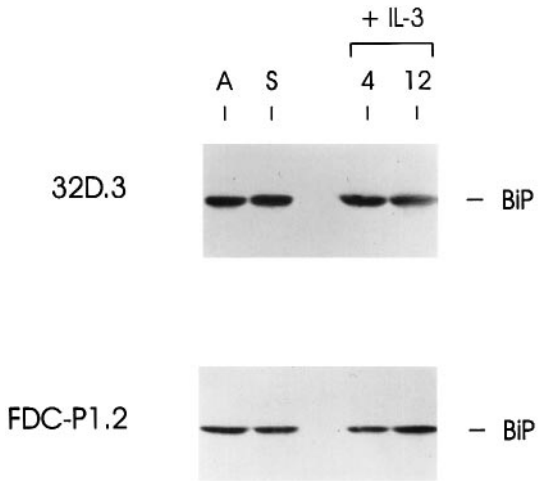

Fig. 7. The steady-state pool of BiP protein is stable during both growth factor deprivation and re-stimulation. Asynchronously growing IL-3-dependent 32D.3 and FDC-P1.2 cells (A) were starved of IL-3 (S) for 16 and 20 h, respectively, and then re-stimulated with IL-3 for the indicated number of hours. The total amount of BiP protein present at each point in the experiment was assessed by Western blotting.

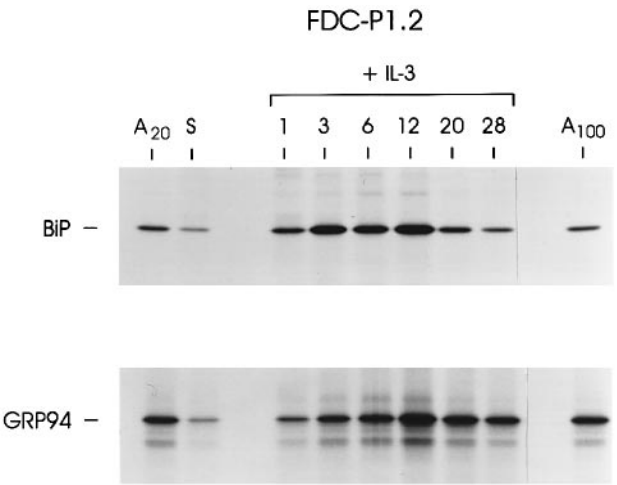

Fig. 8. The rate of ER chaperone biosynthesis increases in response to growth factor stimulation and then returns to normal basal levels. IL-3-dependent FDC-P1.2 cells asynchronously growing in 20 U/ml of IL-3 (A_{20}) were starved of IL-3 for 20 h (S) and then re-stimulated with 100 U/ml of IL-3 for the indicated number of hours. A control asynchronous population was also continuously maintained in 100 U/ml of IL-3 (A_{100}). For each sample, 5×10^6 viable cells were metabolically labeled for 30 min with [^{35}S]methionine and [^{35}S]cysteine. BiP and GRP94 were immunoprecipitated from cell lysates with a rat anti-BiP monoclonal antibody and a rabbit anti-GRP94 polyclonal antiserum respectively. The proteins were resolved by SDS–PAGE under reducing conditions and visualized by fluorography.

FDC-P1.2 cells, as determined by immunoblot analysis, remained stable even after maximal times of IL-3 deprivation (Figure 7). The induction of glycoprotein synthesis in response to IL-3 stimulation exhibited kinetics strikingly similar to those observed for total protein (Figure 6). Therefore, up-regulation of ER chaperones in response to growth factor was not driven by a burst of glycoprotein synthesis early after IL-3 stimulation. These data, taken together with the fact that growth factors do not coordinately induce *CHOP* (Figures 3 and 4), suggested that growth factors regulate the basal expression of ER chaperones via a pathway that is distinct from the UPR.

Because the IL-3 add-back experiments were done with 100 U/ml and continuously cultured cells are maintained in 20 U/ml, we wished to determine if the 2.3-fold increase in BiP biosynthesis at 12 h of IL-3 re-stimulation represented a peak in the kinetics of induction or, alternatively, reflected a higher basal level in the presence of 100 U/ml IL-3. Thus, we monitored the IL-3-mediated induction of both BiP and GRP94 biosynthesis over an extended time course and examined their basal level expression at both 20 and 100 U/ml of IL-3. The rate of translation for both chaperones increased through 12 h after IL-3 re-addition and then returned to the basal level observed in the asynchronously growing population by 20–28 h (Figure 8). Importantly, the basal level translation of both BiP and GRP94 was identical in cells continuously cultured in either the standard concentration of IL-3 (20 U/ml) or the dose used to re-stimulate factor-depleted cells (100 U/ml) (Figure 8). Therefore, the synthesis of ER chaperones following growth factor stimulation peaks at levels greater than their normal basal rates of translation and then equilibrates back to basal levels.

Induction of ER chaperones by growth factors is distinct from the unfolded protein response

To test the hypothesis that growth factors positively regulate expression of ER chaperones by a pathway distinct from the UPR signaling cascade, we directly

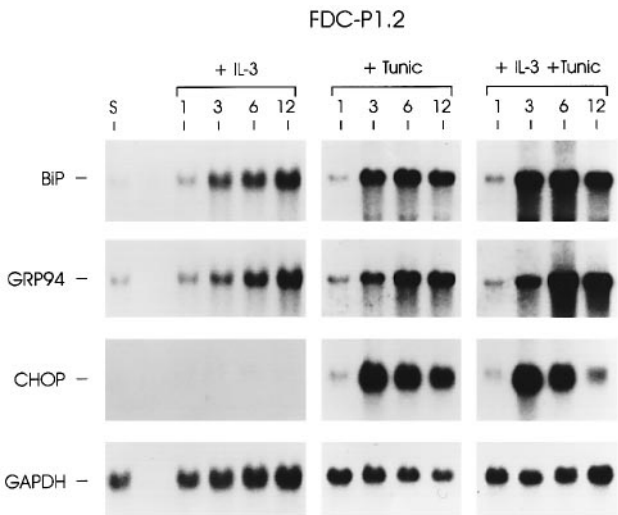

Fig. 9. Growth factors induce ER chaperone genes through a pathway distinct from the unfolded protein response. IL-3-dependent myeloid FDC-P1.2 cells were starved of IL-3 for 20 h (S) and then either re-stimulated with IL-3 (+ IL-3), treated with the glycosylation inhibitor tunicamycin in the continued absence of IL-3 (+ Tunic) or re-stimulated with IL-3 in the presence of tunicamycin (IL-3 + Tunic) for the indicated number of hours. Northern hybridization analyses for *BiP, GRP94, CHOP* and *GAPDH* are shown.

compared the cellular response to growth factors versus tunicamycin, an inhibitor of *N*-linked glycosylation that potently induces the UPR (Lee, 1992). FDC-P1.2 myeloid and FL5.12 pro-B cells were deprived of IL-3 and then treated with either IL-3, tunicamycin or a combination of IL-3 and tunicamycin. As shown for FDC-P1.2 cells in Figure 9 (also see Table II), Northern hybridization analyses revealed the expected induction of *BiP* and *GRP94*

Table II. Fold induction of ER chaperone mRNA in response to IL-3 or tunicamycin

Cell line	mRNA	IL-3		Tunicamycin		IL-3 + Tunicamycin	
		6 h	12 h	6 h	12 h	6 h	12 h
FDC-P1.2	*BiP*	5.9	9.5	20.4	12.5	38.3	20.2
(pro-myeloid)	*GRP94*	3.4	6.4	8.9	7.1	23.0	16.3
	GAPDH	1.7	2.5	1.3	1.0	1.6	2.7
FL5.12	*BiP*	4.3	4.8	12.3	6.4	44.9	19.8
(pro-B)	*GRP94*	1.6	2.7	3.2	1.9	23.6	11.0
	GAPDH	1.1	1.9	0.2	0.1	0.3	0.5

The fold increase (quantitated by PhosphorImager analysis) reflects the change in the amount of mRNA at the indicated timepoints and culture conditions relative to its level after IL-3 deprivation (set at 1).

mRNA in response to IL-3. Cells that had been deprived of IL-3 and that were essentially devoid of basal level ER chaperone transcripts were not compromised in their ability to respond to tunicamycin treatment (Figure 9). Even though translation of secretory pathway proteins had fallen precipitously in factor-depleted cells (Figure 6), tunicamycin robustly induced both *BiP* and *GRP94*, demonstrating that transcription of these genes was possible in the complete absence of essential growth factors. Similarly, *CHOP* was potently induced in IL-3-deprived cells treated with tunicamycin, demonstrating that *CHOP* is coordinately regulated with the ER chaperones via the ER stress pathway in these cells. Importantly, the fact that *CHOP*, *BiP* and *GRP94* were strongly induced by tunicamycin in factor-depleted cells in which the level of nascent secretory pathway proteins entering the ER was greatly reduced (Figure 6) indicates that *CHOP* and the ER chaperone genes do not differ significantly in their sensitivity to ER stress. Finally, when IL-3 and tunicamycin were added together to factor-depleted cells, the two stimuli appeared to synergize in their induction of *BiP* and *GRP94* (Figure 9 and Table II). Together, these data argue that growth factors control transcription of ER chaperone genes via a pathway that is molecularly distinct from the UPR.

Discussion

We have demonstrated that growth factors regulate the expression of ER chaperones. Three lines of evidence indicate that this occurs through a novel pathway that is distinct from the UPR signaling cascade. First, the induction of ER chaperone synthesis after growth factor stimulation was not preceded by a burst of protein traffic through the ER, as determined by monitoring glycoprotein synthesis. Production of some single protein subunits in stoichiometric excess of their partners can activate the UPR (Hendershot *et al.*, 1988; Lenny and Green, 1991). Our data argue that this type of ER stress is not responsible for the up-regulation of BiP and GRP94 synthesis in response to IL-3, since *BiP* induction occurred before, rather than after, glycoprotein synthesis resumed. Second, although growth factor-deprived cells exhibited greatly reduced basal levels of ER chaperone transcripts, a functional and robust UPR could be activated. This was particularly remarkable given that glycoprotein synthesis was reduced in factor-deprived cells to ≈10% of normal levels and tunicamycin exerts its effects exclusively by blocking *N*-linked glycosylation of newly synthesized proteins. This observation illustrates the sensitivity of the UPR and underscores the independent control of the UPR and growth factor-regulated pathways. Indeed, treatment of factor-depleted cells with both growth factor and tunicamycin together appeared to generate a synergistic induction of *BiP* and *GRP94* expression. However, this result may also reflect an enhanced ability of tunicamycin to create ER stress since stimulation of factor-depleted cells with growth factor leads to a gradual increase in glycoprotein synthesis (Figure 6). Finally, the growth factor-regulated and UPR pathways both converge on the ER chaperone genes, but diverge at the level of *CHOP* regulation (Figure 9). To date, growth factor stimulation represents the first example of a stimulus that up-regulates ER chaperone expression without concomitantly inducing *CHOP*. The potent tunicamycin-induced expression of *BiP*, *GRP94* and *CHOP* in factor-depleted cells carrying out only minimal amounts of protein synthesis is in complete agreement with other studies indicating that *CHOP* is triggered by any level of ER stress sufficient to induce ER chaperone genes (Wang *et al.*, 1996; Halleck *et al.*, 1997). Therefore, the absence of *CHOP* induction in growth factor-stimulated cells argues that growth factors regulate expression of ER chaperones via a signaling pathway that is distinct from the stress-inducible UPR.

Although the induction of ER chaperone expression in response to growth factors was fairly rapid, the kinetics were slower than those of c-*myc* induction and required new protein synthesis. Thus, the induction of ER chaperone genes is part of the delayed-early response to mitogenic growth factors. Induction of *BiP* by Epo requires a mitogenically active EpoR and the Jak kinase (Ihle, 1995). However, we also observed growth factor-dependent expression of *BiP* and *GRP94* in colony stimulating factor 1 (CSF-1)-dependent Bac1.2F5 macrophage cells (Table I). The CSF-1 receptor is not coupled to Jak kinases, but rather signals via Src family kinases (Courtneidge *et al.*, 1993). Furthermore, we have also observed growth factor-dependent expression of ER chaperones in serum-dependent NIH-3T3 fibroblasts (data not shown). Thus, the regulation of ER chaperone expression appears to be a general feature of mitogenic signal transduction cascades. We hypothesize that basal transcription of the ER chaperone genes is dependent upon a transcription factor(s) or component(s) of a transcription complex that is regulated through various mitogenic growth factor signaling cascades.

What is the physiological significance of a growth factor-dependent pathway that regulates expression of

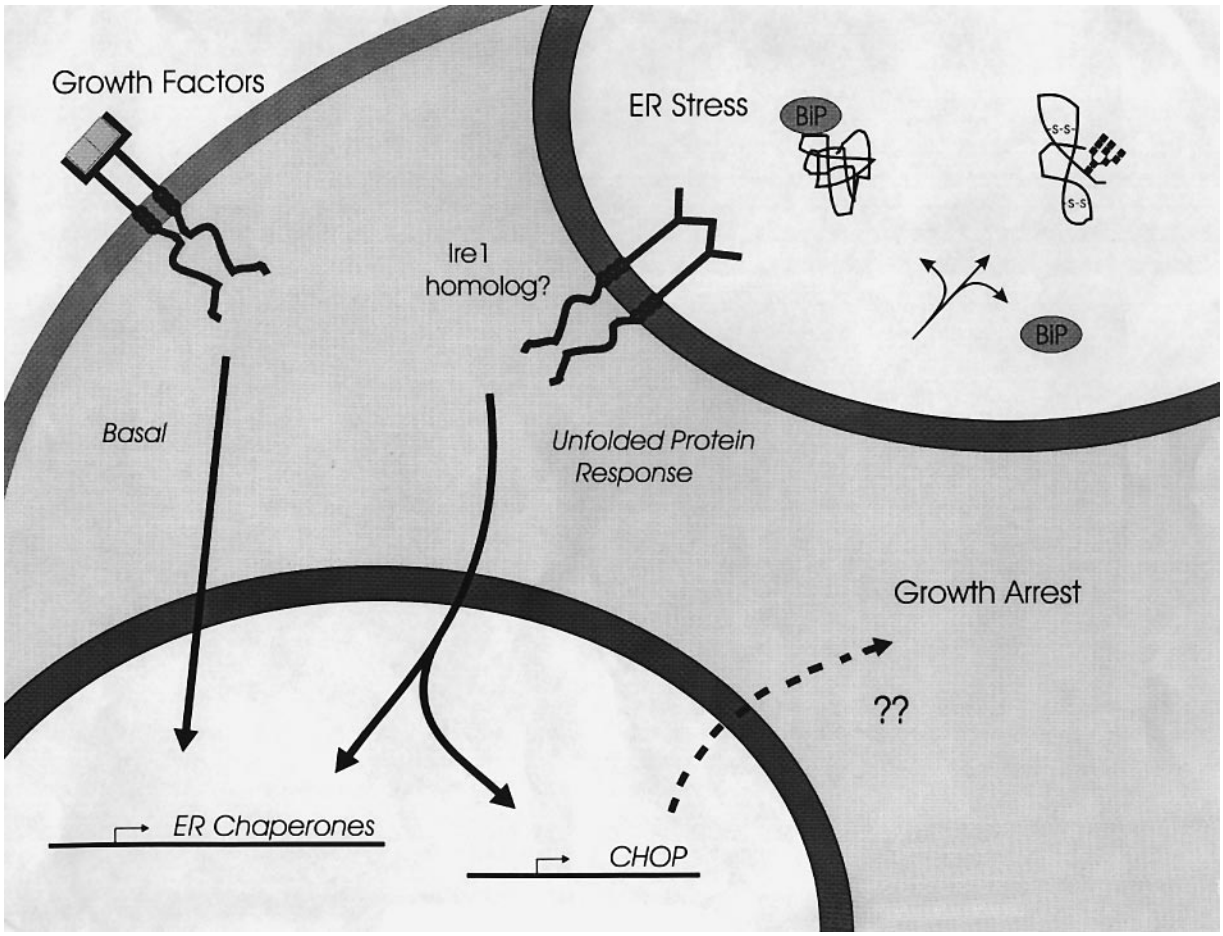

Fig. 10. Two distinct pathways lead to increased biosynthesis of ER chaperones in mammalian cells: the unfolded protein response and growth factor stimulation. In mammalian cells, the accumulation of unfolded proteins bound to BiP in the ER (for example, due to tunicamycin treatment) generates a stress signal, perhaps via an ER transmembrane kinase (Ire1p homolog), that leads to the coordinated up-regulation of ER chaperone gene expression and induction of the transcription factor CHOP. CHOP may be involved in the arrest of cell growth. By contrast, mitogenic growth factors activate signaling cascades that result in cell proliferation and induce expression of ER chaperones but not of CHOP. We propose that this pathway allows cells to maintain basal expression of ER chaperones and, perhaps, to up-regulate their expression in response to growth and/or differentiation signals without activating the stress-inducible UPR cascade.

ER chaperones? Our data strongly imply that the basal expression of genes encoding ER chaperones does not reflect a constant low level of ER stress that weakly activates the UPR. Rather, basal transcription of ER chaperone genes appears to be a regulated process controlled by signals delivered via various growth factor receptors in normal growing cells (Figure 10). This is in agreement with the fact that the yeast UPR is dispensable under normal, non-stressed conditions (Cox *et al.*, 1993; Mori *et al.*, 1993). Our model predicts that basal and stress-inducible expression may be mediated by distinct elements within the promoters of ER chaperone genes such as *BiP*. While this has not yet been demonstrated for the mammalian *BiP* promoter, there is evidence for separate control of basal and stress-inducible activity of the yeast *KAR2* promoter (Mori *et al.*, 1992; Kohno *et al.*, 1993).

Our results also suggest that cells possess at least two distinct pathways that lead to up-regulated synthesis of ER chaperones and that these pathways are activated for strikingly different, yet physiologically relevant, reasons: growth stimuli versus stress in the ER (see Figure 10). Importantly, both pathways converge on the ER chaperone genes, but differ in their effect on other UPR targets such as the CHOP transcription factor. We emphasize that it remains to be determined whether growth arrest and/or cell death is a direct or indirect effect of stress in the ER, and there are limited data concerning the potential role of CHOP in these events. However, it is intriguing to speculate that the UPR, being a stress-activated pathway, induces the synthesis of the protective ER chaperones and folding enzymes together with other gene products, for example CHOP, that might be involved in arresting growth or initiating apoptosis in response to ER stress. By contrast, the growth factor-regulated pathway induces synthesis of ER chaperones while bypassing other genes such as *CHOP* that are coordinately induced by the UPR (see Figure 10). In this way, cells stimulated by growth factors, and perhaps also by differentiation factors (Wiest *et al.*, 1990; Nakai *et al.*, 1995), can increase production of ER chaperones and folding enzymes to accommodate enhanced levels of protein traffic through the ER without activating genes that may be deleterious under normal, non-stressed conditions.

The ER is a dynamic organelle, serving as both a specialized compartment for the folding and assembly of secretory pathway proteins and as a sensor for certain types of cellular stress. The stressed ER is linked, at least, to the UPR pathway as well as to the activation of the NF-κB transcription factor complex (Pahl and Baeuerle,

1995). We have shown that growth factors positively affect the ER by stimulating expression of ER chaperones under normal conditions. To our knowledge, this represents the first signaling pathway identified in mammalian cells that modulates expression of the ER chaperones independently of stress conditions in the ER.

Materials and methods

Cells and cell culture

The IL-3-dependent myeloid cell lines, 32D.3 and FDC-P1.2, have been described previously (Dean *et al.*, 1987; Askew *et al.*, 1991). IL-3-dependent myeloid FL5.12 cells were kindly provided by Dr Gabriel Nuñez (University of Michigan, Ann Arbor, MI). The IL-3-dependent myeloid DA-3 cell line and the DA-3/EpoR derivatives were provided by Dr James Ihle (St. Jude Children's Research Hospital). IL-3-dependent cell lines were maintained in RPMI-1640 with 20 U/ml of recombinant IL-3. The CSF-1-dependent Bac1.2F5 macrophage cell line was provided by Dr Charles Rock (St. Jude Children's Research Hospital) and maintained in L cell medium which contains CSF-1. The c-*myc*-overexpressing 32D.3 cells and neo controls have been described previously (Askew *et al.*, 1991). The Bcl-2-overexpressing 32D.3 cells were generated using the SFFV-hBcl-2 mammalian expression vector provided by Dr Stanley Korsmeyer (Washington University, St. Louis, MO). FL5.12 cells overexpressing Bcl-X_L were generously provided by Dr Gabriel Nuñez. Neo controls were generated using the SFFV vector provided by Dr Stanley Korsmeyer. Transfection by electroporation followed by selection in G418 was carried out as previously described (Hiebert *et al.*, 1995).

For growth factor deprivation experiments, cells were washed twice and then cultured in the absence of growth factor for the intervals indicated in the figure legends. Growth factor deprivation of individual cell lines yielded 80–90% viable cells, as determined by trypan blue dye exclusion, and 70–80% of cells in G_0/G_1 arrest, as determined by propidium iodide staining and flow cytometry. Stimulation of IL-3-deprived cells was carried out with 100 U/ml recombinant IL-3 and with 3 U/ml Epo for DA-3/EpoR transfectants, as indicated in the figure legends. To block protein synthesis during growth factor stimulation, cycloheximide (Sigma, St. Louis, MO) (50 μg/ml) was added to the culture medium 15 min prior to addition of growth factor and remained for the duration of the experiment as indicated. To block *N*-linked glycosylation, cells were treated with tunicamycin (Sigma) (10 μg/ml) as indicated.

Isolation and analysis of RNA

Total RNA was extracted from cultured cells and analyzed by Northern blotting as described previously (Cleveland *et al.*, 1989). The probe for BiP was a 1.5 kb *Eco*RI–*Pst*I fragment from a hamster cDNA clone provided by Dr Amy S.Lee (USC, Los Angeles, CA). The probe for GRP94 was a 1.0 kb *Sal*I–*Hin*dIII fragment from a mouse GRP94 cDNA provided by Dr Michael Green (St. Louis University, St. Louis, MO). The probe for OST48 was a 750 bp *Pst*I fragment from a canine cDNA provided by Dr Reid Gilmore (UMASS Medical Center, Worcester, MA) The probe for CHOP was a 600 bp *Eco*RI–*Xba*I fragment from a mouse cDNA provided by Dr David Ron (NYU, New York, NY). The probes for actin and c-Myc have been previously described (Cleveland *et al.*, 1989). A Fli-1 cDNA fragment was provided by Dr James Ihle. A murine Max cDNA fragment was provided by Dr Edward Ziff (NYU). A 1 kb murine cDNA fragment for GAPDH was provided by Dr Thomas Look (St. Jude Children's Research Hospital). Probes were prepared by random primer labeling using the Prime It II kit (Stratagene, La Jolla, CA) and [α-^{32}P]dCTP (Amersham, Arlington Heights, IL). Hybridization was performed as described (Cleveland *et al.*, 1989). Hybridization signals were analyzed by autoradiography and PhosphorImaging (Molecular Dynamics, Sunnyvale, CA) using ImageQuant Software as described in the figure legends and tables.

Biosynthetic labeling

To determine total protein synthesis rates, cells (5×10^6/ml) were incubated in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD) lacking methionine and cysteine and containing 50 μCi/ml Tran[^{35}S]-label (ICN, Irvine, CA). Cell lysates were prepared and proteins were immunoprecipitated as described previously (Bole *et al.*, 1986). BiP was precipitated with a rat monoclonal antibody (Bole *et al.*, 1986). GRP94 was precipitated with a rabbit polyclonal antibody previously described (Wang *et al.*, 1996). Immune complexes were captured with protein A–Sepharose (Sigma). Proteins were electrophoresed on 10% SDS–polyacrylamide gels. Labeled proteins were visualized by fluorography using the Amplify reagent (Amersham) and by PhosphorImaging. To monitor protein traffic in the secretory pathway, the rate of glycoprotein synthesis was determined. Cells (5×10^6/ml) were incubated in low glucose (0.2 mg/ml) RPMI (Life Technologies) containing 10 μCi/ml D-[2-^{3}H(N)]mannose (DuPont NEN, Boston, MA). Incorporation of radiolabeled amino acids or mannose was determined by measuring trichloroacetic acid (TCA)-precipitable activity in labeled cell lysates. Equivalent amounts of cell lysates were spotted on Whatman filter paper in duplicate. Filters were boiled in 10% TCA and analyzed by scintillation spectroscopy.

Western blotting

Cell lysates were prepared in RIPA buffer and protein concentrations were determined using a Bradford assay. Equivalent amounts of total protein were resolved by 10% SDS–PAGE and BiP was detected by Western blotting using a monoclonal rat anti-mouse BiP antibody (Bole *et al.*, 1986), followed by a goat anti-rat IgG (Southern Biotechnology Associates, Birmingham, AL) and horseradish peroxidase-conjugated protein A (EY Laboratories, San Mateo, CA), and developed by ECL (Amersham).

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