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Hormone-conditional transformation by fusion proteins of c-Abl and its transforming variants

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Fusion of the hormone binding domain (HBD) of steroid receptors to transcription factors renders them hormone-dependent. We show here that an SH3-deleted, oncogenic variant of the Abl tyrosine kinase becomes hormone-dependent for transformation by fusion to the estrogen receptor (ER) HBD, extending the phenomenon to tyrosine kinases. Surprisingly, fusion of the HBD to the normal, non-transforming c-Abl (IV) protein activated transforming activity in a hormone-dependent fashion. In the presence of hormone, the c-Abl:ER fusion protein was transforming, cytoplasmic and tyrosine phosphorylated, whereas it was non-transforming, nuclear and hypophosphorylated without hormone. We have examined the kinetics of activation of the c-Abl:ER protein and found that protein synthesis is required both for kinase activation and for redistribution of the c-Abl:ER protein from the nucleus to the cytoplasm. We suggest that the activation of c-Abl could be due to HBD-mediated dimerization and/or to the ability to overexpress conditionally the normally toxic c-Abl protein. This novel approach may be applicable to a wide variety of proteins, particularly when activating mutations or physiological inducers are unknown or when the protein is toxic to cells.

Key words: Abl/conditional mutations/hormone binding domain/estrogen receptor/fibroblast transformation/tyrosine kinase

Introduction

Protein tyrosine kinases have been intensively studied for their roles in growth regulation, differentiation, and oncogenesis. For the transmembrane tyrosine kinases with identified ligands, such as the receptors for epidermal growth factor, platelet-derived growth factor, colony stimulating factor 1 and fibroblast growth factor, both the inductive signal and aspects of the mechanistic control of the kinase activity are partially understood (Yarden and Ullrich, 1988). Notably, these receptors are thought to be activated by growth factor-induced aggregation or dimerization (Ullrich and Schlessinger, 1990). However, for the non-receptor tyrosine kinases of the Src, Abl and Fps families, the inductive signals and mechanisms are less clear, making their normal physiology difficult to study. Only recently have potential growth factor signals and regulatory mechanisms been suggested for members of the Src family, notably c-Lck, in the context of T cell signaling (Bolen and Veillette, 1989; Hatakeyama *et al.*, 1991). These non-receptor tyrosine kinases have been studied largely as transforming variants, which may only partially mirror their normal physiological roles. Clearly, the possibility of regulating manipulated variants of these kinases by exogenous signals that do not perturb normal physiology could help to assess downstream events.

Control of transforming processes dependent on non-receptor tyrosine kinases has been accomplished using temperature-sensitive mutants of transforming genes, such as *src* or *abl* (Anderson *et al.*, 1987; Engelman and Rosenberg, 1987; Kipreos *et al.*, 1987). However, for *in vivo* studies in mammalian systems, such as the investigation of Abl-induced leukemia in mice, temperature-sensitive mutations are of limited utility. In the case of transmembrane receptors, functional substitution of a heterologous extracellular domain for that normally associated with the tyrosine kinase has also been reported and may be useful for defining the role of a particular receptor without using the natural ligand (see, for example, Seedorf *et al.*, 1991; Yan *et al.*, 1991, and references therein).

Recently, regulable variants of several transcription factors have been constructed by fusion to the hormone binding domains (HBDs) of the glucocorticoid receptor or the estrogen receptor (ER). These regulatory domains can inactivate the heterologous activity in a fashion that can be reversed by hormone (Picard *et al.*, 1988; Eilers *et al.*, 1989; Burk and Klempnauer, 1991; Superti-Furga *et al.*, 1991; Umek *et al.*, 1991). It has been proposed that the inactivation is mediated by the hormone-reversible association of the HBD with a heat shock protein, HSP90 (Picard *et al.*, 1988). We now report that this strategy can also be applied to a tyrosine kinase. Thus, a transforming derivative of c-*abl* becomes a hormone-inducible oncogene by gene fusion to a region encoding a steroid binding domain. Surprisingly, we discovered that a normal, non-transforming c-Abl could be activated by fusion to the HBD of the ER. In the presence of hormone, this hybrid protein becomes a potent transforming protein despite being composed of two oncogenically inactive moieties. We use this hormone-regulable Abl kinase to study the kinetics of kinase activation, to identify a protein synthesis requirement for kinase activation, to correlate the formation of a high molecular weight complex with Abl-dependent transformation and to show transformation-dependent redistribution of the Abl protein from the nucleus to the cytoplasm.

Results

Construction of hormone-regulable derivatives of the Abl kinase

To construct hormone-regulable mutants of the Abl protein, we examined whether the regulation of transforming activity by the ER HBD observed for transcription factors could be extended to a transforming variant of Abl.

Hormone-reversible inactivation of transforming Abl by fusion to a steroid receptor hormone-binding domain. To express a hormone-inducible derivative of Abl, we constructed a retroviral vector, pPL, encoding a fusion protein, denoted by ΔXB:ER, including the N-terminal domains of the ΔXB transforming mutant of c-Abl (IV) fused to the human ER HBD (Figure 1). The original ΔXB mutant had a deletion of the Src homology domain 3 (SH3) of the non-transforming c-Abl (IV) and possessed the same transforming properties as v-Abl (Jackson and Baltimore, 1989). The C-terminal domain of Abl has several other potential functions including DNA binding, actin association and a role in some transformation assays, but is not required for fibroblast transformation (Prywes *et al.*, 1985; Van Etten *et al.*, 1989; Kipreos and Wang, 1992). Thus, to simplify our analysis, the domain C-terminal to the major nuclear localization signal was deleted in the ΔXB:ER fusion protein. As a control for this deletion, we constructed a similar C-terminal deletion mutant of ΔXB without fusion to the HBD, ΔXB-Δ630 (Figure 1). Retroviral vectors encoding these mutants and the parental c-Abl and ΔXB mutants were cotransfected with a cloned Moloney helper virus into NIH 3T3 cells, which were plated with and without estradiol.

The ΔXB:ER and ΔXB-Δ630 constructs produced similar

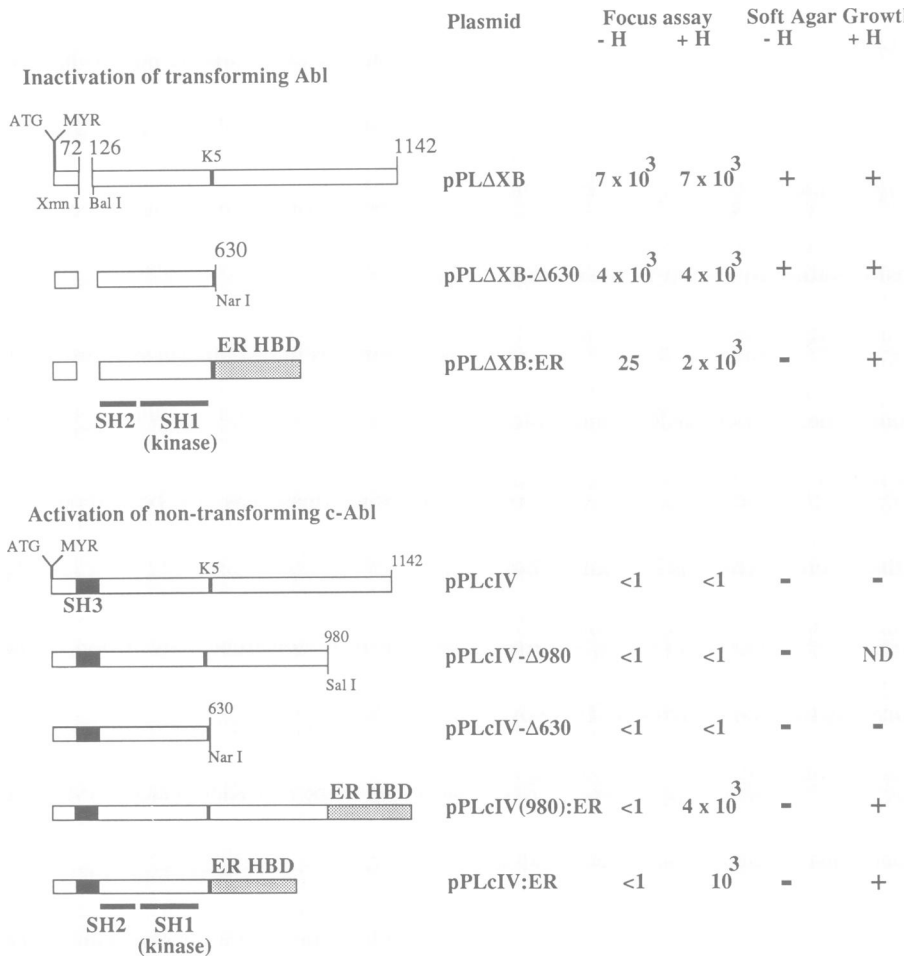

Fig. 1. Schematic representations and biological properties of Abl protein derivatives fused to the ER HBD. c-Abl sequences and ER HBD are represented by open and shaded boxes, respectively. Several c-Abl characteristics are indicated: ATG, initiator codon; MYR, myristoylation site; SH1, SH2 and SH3, Src homology domains 1, 2 and 3, respectively; K5, the pentalysine motif of the c-Abl nuclear localization signal. Landmark restriction sites used for deletion are indicated. Note that transforming Abl variants lack SH3. (**Above**) Dominant inactivation of transforming Abl. pPLΔXB: an SH3 deletion mutant of c-Abl (IV) (Jackson and Baltimore, 1989). pPLΔXB-Δ630: a double mutant of c-Abl (IV) with both the SH3 deletion and the C-terminal deletion of residues 631−1142. pPLΔXB:ER: a fusion protein with the SH3 deletion mutant of c-Abl (IV), ΔXB-Δ630, and residues 282−595 of the human ER. (**Below**) Activation of normal, non-transforming c-Abl. pPLcIV: a retroviral vector encoding full-length c-Abl (IV) (Jackson and Baltimore, 1989). pPLcIV-Δ980: a derivative of pPLcIV, encoding c-Abl (IV) with C-terminal residues 981−1142 deleted. pPLcIV-Δ630: a derivative of pPLcIV, encoding c-Abl (IV) with C-terminal residues 631−1142 deleted. pPLcIV(980):ER: a fusion protein containing residues 1−980 of c-Abl (IV) and residues 282−595 of the human ER. pPLcIV:ER: a fusion protein containing residues 1−630 of c-Abl (IV) and residues 282−595 of the human ER. Constructs were cotransfected with a cloned M-MuLV provirus (pZAP) into NIH 3T3 cells. On day 2, the transfected cells were split into media with or without 1 μM estradiol (+H or −H). Transformed foci were counted on day 10 post-transfection. 'Transformation' refers to the number of morphologically transformed foci per plate (normalized for the dilution when split). Populations of transfected cells were plated in 0.48% Difco agar in DME with 10% donor calf serum either with or without 1 μM β-estradiol. 'Soft agar growth' was scored by counting macroscopic colonies at 21 days.

numbers of morphologically transformed foci in the presence of hormone, but the ΔXB:ER construct produced ~80-fold fewer foci in the absence of hormone. The foci formed in the absence of hormone were small and could not be passaged as cell lines without the addition of hormone. This failure of lines to grow without hormone is probably due to a strong cytostatic effect of the Abl:ER fusion protein in the absence of hormone (P.Jackson, D.Baltimore and D.Picard, in preparation). Further, the cells transformed by the ΔXB:ER construct failed to grow without hormone in the more stringent soft agar growth assay, but did form colonies in agar in the presence of estradiol (Figure 1). The ΔXB-Δ630 mutant transformed cells only slightly less well than the parental ΔXB mutant, and these mutants were not affected by hormone in either the focus formation or soft agar growth assays (Figure 1). Thus, the ER HBD inactivated the transforming ability of the ΔXB mutant in a hormone-reversible fashion. Similar results were obtained with the HBD of the glucocorticoid receptor (data not shown).

Oncogenic activation of non-transforming c-Abl by fusion to the ER hormone binding domain. Initially intended as a negative control, we constructed a vector encoding a c-Abl:ER hybrid protein retaining the SH3 domain, called cIV:ER (Figure 1). We were surprised to find that this construct also transformed NIH 3T3 cells. While no foci appeared in the absence of hormone, the cIV:ER construct transformed quantitatively almost as well as the ΔXB:ER or ΔXB-Δ630 constructs in the presence of hormone (Figure 1). As expected, the C-terminal deletion mutant cIV-Δ630 did not transform with or without estradiol, demonstrating that the simple deletion of the C-terminus did not activate the transforming ability of c-Abl (IV). Thus, the HBD of the human ER activated c-Abl (IV) in the presence of hormone.

A different c-Abl:ER hybrid protein including more of the Abl C-terminus, cIV(980):ER (Figure 1) was also activated for transformation in the presence of hormone. Thus, the ER HBD will function in different contexts within the c-Abl protein to activate transforming ability.

Abl:ER fusion proteins with and without SH3 transform reversibly, but with different kinetics

We examined whether the transforming effect of the Abl:ER fusion proteins was reversible in stable, clonal cell lines. Populations of NIH 3T3 cells transformed by the cIV:ER and ΔXB:ER mutants were grown in estradiol and cloned by limiting dilution. In multiple clonal NIH 3T3 lines expressing the cIV:ER fusion protein, cells grown in the

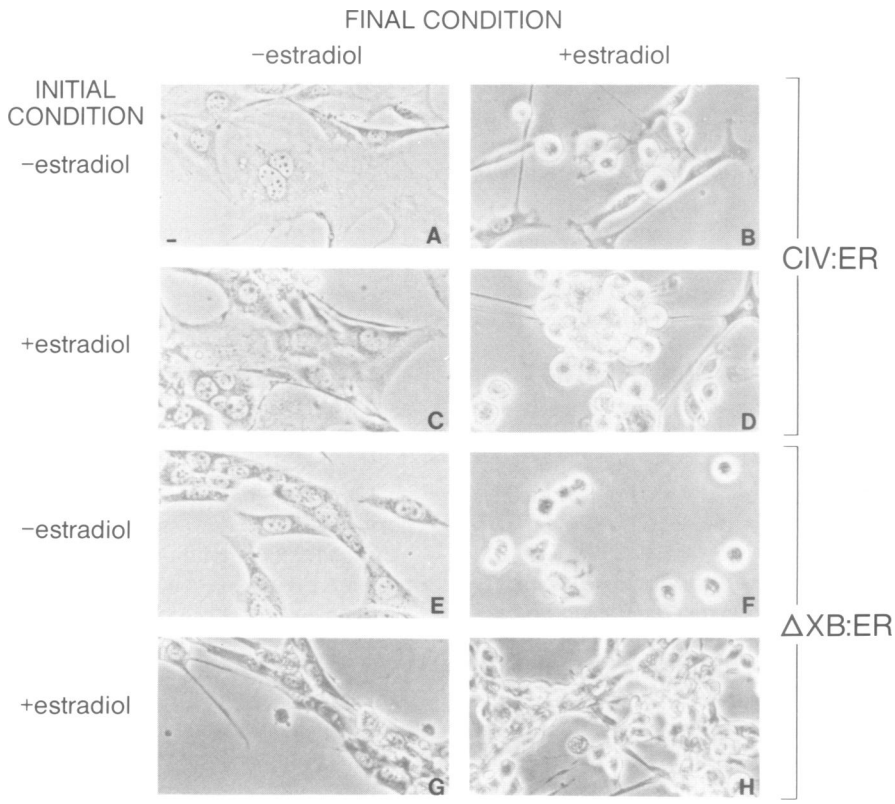

Fig. 2. Hormone-dependent morphological transformation. Clonal lines expressing the $p103^{cIV:ER}$ and $p96^{\Delta XB:ER}$ fusion proteins were grown in the presence of estradiol (1 μM) in a morphologically transformed state. Cells were washed and plated either with or without estradiol and grown for 2 days (INITIAL CONDITION). Plates were then washed and fed with or without estradiol, and grown for an additional 2 days (FINAL CONDITION). (**A**–**D**) NIH 3T3 cells expressing $p103^{cIV:ER}$. (**E**–**H**) NIH 3T3 cells expressing $p96^{\Delta XB:ER}$. (A and E) Cells grown for 2 days without estradiol reverted fully and maintained their flat morphology. (B and F) Cells were grown for 2 days without estradiol and reverted fully. These cells were treated with estradiol and grown for an additional 2 days. Much of the fully transformed morphology was seen at day one. (C and G) Cells in estradiol showing a fully transformed morphology were washed and refed with medium lacking hormone and grown for an additional 2 days. The reversion is complete with the cIV:ER cells, while the reversion of the ΔXB:ER cells is substantial, but slower and more variable. (D and H) Cells that have been grown for 4 days in estradiol maintain their transformed morphology and grow considerably faster than control cells without hormone. Scale: Nuclear diameter in photomicrograph A is ~10 μm.

presence of estradiol were highly morphologically transformed. When viewed by phase contrast microscopy, the cIV:ER cells were homogeneously round and refractile, grew to high density without apparent contact inhibition, and, when viewed by time-lapse video microscopy, were highly motile with rapidly changing, extended cellular processes and reduced lamellipodia. In the absence of hormone, the cIV:ER cells were uniformly flat, showed normal, non-refractile cytoplasmic and phase-dark nuclear structure, low motility and ruffling lamellipodia. In the absence of hormone, the ΔXB:ER cells behaved similarly, but were less homogeneously flat, with some cells showing intermediate morphologies (data not shown).

When clonal lines that had been transformed in the presence of hormone by the cIV:ER mutant were washed and plated in the absence of hormone (3600 cells/cm^2), most of the cells showed a flat morphology within 1 day. By the second day, the cells had homogeneously reverted (Figure 2). The rate of reversion was somewhat lower at high cell density (>26 000 cells/cm^2). Adding hormone to cells that had reverted induced their morphological transformation (Figure 2). During the time course following hormone addition, intermediate transformed morphologies were recognizable, but within 2 days the cells were morphologically indistinguishable from cells grown continuously in hormone or from NIH 3T3 cells transformed by v-Abl (Figure 2 and data not shown). Thus, the transformed phenotype was reversibly dependent on the continued presence of estradiol.

Cells transformed by the ΔXB:ER fusion protein required more time to revert homogeneously after removal of hormone than the cIV:ER expressing cells, but nonetheless showed substantial reversion within 2 days. Reverted cells were induced to a transformed phenotype by addition of hormone within 1 day (Figure 2). Cells with the ΔXB:ER fusion protein apparently reverted with more difficulty and were induced more easily than cells with the cIV:ER fusion protein, suggesting that the ΔXB:ER fusion protein had higher levels of transforming activity in cells. Growth of the cIV:ER expressing lines in soft agar was also somewhat slower than that of the ΔXB:ER-expressing lines in the presence of hormone. Thus, the transforming activities of both the ΔXB:ER and cIV:ER fusion proteins were hormone-dependent and reversible, but with different kinetics of morphological response to hormone.

Abl:ER fusion proteins with and without SH3 have different hormone dosage responses for hormone-induced transformation

Because the cIV:ER- and ΔXB:ER-expressing lines had different kinetic responses to hormone, we examined the dose-dependence for growth in soft agar. The ΔXB:ER fusion protein responds half-maximally to hormone at concentrations slightly higher than the known K_d of the HBD for estradiol (see Table I) and consistent with the dosage response of a myc:ER fusion protein (Eilers *et al.*, 1989). In contrast, half-maximal stimulation of the cIV:ER fusion protein required ~20-fold more hormone (Table I). Both showed a sigmoidal response to varying concentrations of hormone (data not shown). Thus, there were differences in hormone responsiveness of the cells expressing the Abl:ER fusion proteins with or without the SH3 domain.

To address the possibility that the Abl:ER fusion proteins with or without SH3 had different affinities for hormone, we performed *in vivo* hormone binding studies. Cells expressing the cIV:ER fusion protein bound [^{3}H]estradiol with ~5-fold lower affinity than the ΔXB:ER cells (Table I). Both fusion proteins expressed several million hormone binding sites per cell, consistent with high levels of Abl:ER fusion protein expression (>10-fold more than endogenous c-Abl). Although the cIV:ER fusion protein has a lower affinity for ligand than the ΔXB:ER fusion protein, this difference cannot account for the 20-fold difference in hormone level required for transformation. Possibly, the difference in behavior of the two fusion proteins could be due to an inhibitory function of SH3 in c-Abl (Franz *et al.*, 1989; Jackson and Baltimore, 1989; Pendergast *et al.*, 1991; Cicchetti *et al.*, 1992) or to differences in subcellular localization of the two fusion proteins (see below).

Table I. Hormone binding and induction of Abl:ER fusion proteins

Cell line	Number of molecules per cell	Hormone-binding affinity (K_d)[a]	Half-maximal dose for growth in soft agar[b] (nM)
ΔXB:ER	4.2×10^6 ± 24%	2.5 nM ± 18%	~8
cIV:ER	2.0×10^6 ± 14%[c]	13.3 nM ± 24%	~150

[a]Affinity for β-estradiol.
[b]Estimated by graphical analysis of the β-estradiol dose–response curve for growth in soft agar.
[c]This is a minimum estimate because these cells were less adherent in serum-free medium and some cells were unavoidably lost during washing. Loss of cells during washing would not affect the measured affinity, but might cause the number of binding sites per cell to be underestimated.

The transforming activities of the Abl:ER fusion proteins correlate with hyperphosphorylation on tyrosine

A characteristic biochemical difference between *abl*-transformed cells and parental cells is the presence of specific proteins phosphorylated on tyrosine residues, including Abl itself. We were interested in whether induction of tyrosine phosphorylation of these proteins occurred in cells expressing the Abl:ER fusion proteins after hormone addition. Probing an immunoblot for Abl protein showed that addition of hormone to reverted cells caused a slight reduction in the electrophoretic mobility for the 103 kDa cIV:ER fusion protein (Figure 3). An immunoblot performed in parallel using an anti-phosphotyrosine antibody showed that the less mobile form of the cIV:ER doublet seen in the presence of hormone is tyrosine phosphorylated, while in the absence of hormone, the more mobile form did not appear to be tyrosine phosphorylated (compare panels in Figure 3). Similarly, in cells expressing the 96 kDa ΔXB:ER fusion protein in the presence of hormone, this protein appeared to be hyperphosphorylated. In the absence of hormone, the ΔXB:ER protein appeared significantly less tyrosine phosphorylated (~5-fold) but did maintain some basal tyrosine phosphorylation. In some experiments, a subtle size shift dependent on hormone was apparent for the ΔXB:ER protein also (data not shown). Thus, the transforming forms of the Abl:ER fusion proteins had higher levels of tyrosine phosphorylation than the non-transforming forms.

We also examined the *in vitro* kinase activity of the Abl:ER

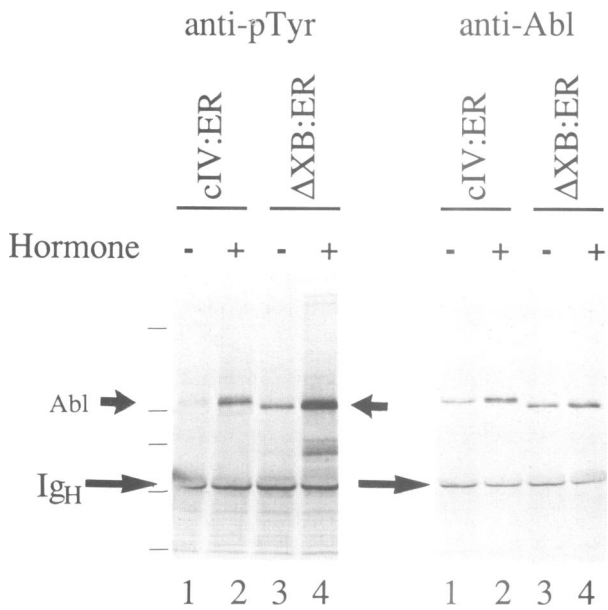

Fig. 3. Hormone-dependent induction of Abl-dependent phosphorylation. Clonal lines expressing the cIV:ER and ΔXB:ER fusion proteins were washed, plated without estradiol and grown for 2 days to allow the cells to revert. Plates of reverted cells were treated with hormone or mock treated for 2 days. Extracts were immunoprecipitated with antiserum specific for c-Abl (IV), run on 5−15 % SDS−polyacrylamide gradient gels, blotted on to Immobilon (Millipore), and stained for anti-phosphotyrosine or Abl determinants (see Materials and methods). Lanes 1, cIV:ER cells without hormone; lanes 2, cIV:ER cells with 1 μM estradiol; lanes 3, ΔXB:ER cells without hormone; lanes 4, ΔXB:ER cells with 1 μM estradiol. **Left panel**: anti-phosphotyrosine Western blot shows an increase in the level of Abl tyrosine phosphorylation (compare with right panel for normalization) from low basal levels to high levels for cIV:ER-expressing cells and from moderate basal levels to high levels for ΔXB:ER-expressing cells. **Right panel**: anti-Abl Western blot. Note the comigration of the upper species with the hyperphosphorylated form of Abl for cIV:ER. Abl species are indicated with short arrows and the heavy chain of the precipitating antibody with a long arrow. Molecular weight markers on the left are 30, 46, 69, 97.4 and 200 kDa.

proteins in an immune complex kinase assay and found no apparent hormonal effect. Both cIV:ER and ΔXB:ER proteins had levels of kinase activity proportional to the amount of protein immunoprecipitated from cells treated either with or without hormone (not shown) suggesting that the *in vitro* kinase assay did not reflect the *in vivo* activity. Under the conditions of the assay, interaction with HSP90, which might be required for any hormone-dependent regulation, would not be maintained. Direct addition of hormone to the immune complex kinase assay also had no apparent effect (not shown).

The SH3 domain delays the induction of tyrosine phosphorylation

We examined the kinetics of activation of phosphorylation of cellular proteins by anti-phosphotyrosine Western analysis. Clonal lines expressing the cIV:ER and ΔXB:ER fusion proteins were plated in the absence of hormone and allowed to revert for 2 days. Estradiol was added to 1 μM (t = 0) and lysates were prepared at various times and examined by anti-Abl and anti-phosphotyrosine Western analysis.

The cIV:ER mutant showed a subtle increase in Abl protein level over 24 h, but a more dramatic increase in autophosphorylation within 1−2 h and then transphosphorylation of previously noted cellular proteins within one to several hours (Figure 4A and C). These tyrosine phosphorylated species are identical to those seen in NIH 3T3 cells transformed by v-Abl or an SH3-deleted c-Abl (species indicated are 36, 62, 85, 210/220 and 280−300 kDa). The general kinetics of activation of the cIV:ER fusion protein was reproducible but showed some variation in the time required to show strong anti-phosphotyrosine staining (2−6 h).

The ΔXB:ER mutant showed a different kinetics of activation. Both the autophosphorylation/size shift of the ΔXB:ER Abl species and the transphosphorylation of the cellular species occur in < 10 min (Figure 4B and D). In contrast, an increase in the level of the ΔXB:ER protein requires > 10 h. Thus, the activation of the ΔXB:ER protein by hormone addition occurs with faster kinetics than that for the activation of the cIV:ER protein.

The background level of tyrosine phosphorylated species in the ΔXB:ER expressing cells was higher in the absence of hormone than for the cIV:ER cells, suggesting that the ΔXB:ER protein has some kinase activity without hormone. However, phosphorylation of these background species is not induced by hormone, suggesting that they are not relevant substrates for transformation. In contrast, the phosphorylation of some species like the 62 kDa protein is highly dependent on hormone (Figure 4D), suggesting that these may be crucial substrates for transformation.

Protein synthesis is required for activation of cIV:ER, but not ΔXB:ER

When reverted cells were pretreated with cycloheximide for 30 min before addition of hormone, the level of the $p103^{cIV:ER}$ protein decreased only slowly over the 18 h time course as seen by Western blotting (Figure 5A). Inspection of an anti-phosphotyrosine Western blot showed no activation of phosphotyrosine-containing proteins by the Abl kinase in the presence of cycloheximide (Figure 5C).

Cells expressing the SH3-deleted Abl:ER fusion protein, $p96^{ΔXB:ER}$, did show considerable induction of tyrosine phosphorylation of characteristic species, even in the presence of cycloheximide (Figure 5D). Furthermore, the level of phosphotyrosine in these proteins was maintained in the absence of protein synthesis for at least several hours (Figure 5B and D). This suggests that cycloheximide blocks activation of the tyrosine kinase activity of the existing pool of cIV:ER fusion protein, and rules out a failure to maintain tyrosine phosphorylated proteins in the absence of protein synthesis. This requirement for protein synthesis could reflect the need to synthesize either new cIV:ER protein or proteins required for Abl activation, or both. Cycloheximide apparently inhibited the ability to induce morphological transformation for both cIV:ER and ΔXB:ER lines, but the toxicity of the drug on this time scale made this experiment difficult to interpret.

Immunofluorescence shows hormone-induced redistribution of cIV:ER from nucleus to cytoplasm

Our previous work suggested a difference in subcellular localization between normal c-Abl (IV) [which was partly nuclear and partly associated with actin-rich stress filaments

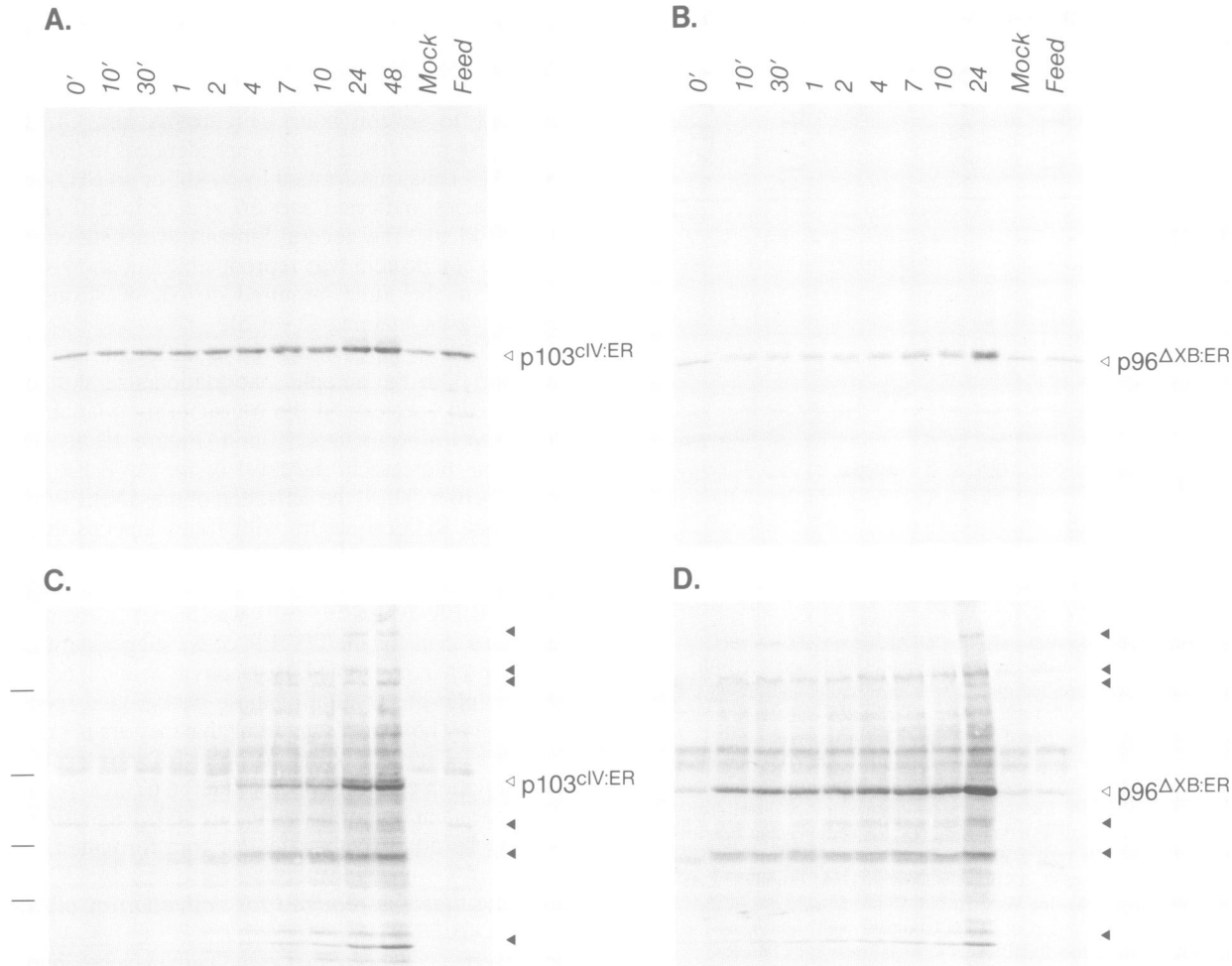

Fig. 4. Kinetics of estrogen-induced tyrosine phosphorylation. Clonal lines expressing the cIV:ER and ΔXB:ER fusion proteins were washed, plated without estradiol and grown for 2 days in order to revert the cells morphologically. Multiple plates of reverted cells were treated with hormone and extracts prepared at the indicated times. The lane labeled 'mock' indicates an equivalent plate with no treatment and the lane labeled 'feed' was refed with fresh medium without hormone. Extracts were run on 5–15% SDS–polyacrylamide gradient gels and analyzed by anti-Abl and anti-phosphotyrosine Western blotting as described in Materials and methods. (**A** and **C**) Time course of estrogen induction of cIV:ER. (**B** and **D**) Time course of estrogen induction of ΔXB:ER. (A and B) Anti-Abl Western analysis shows the p103 and p96 Abl species using a monoclonal antibody (mAb 19-84) against the kinase domain of Abl. (C and D) Anti-phosphotyrosine Western analysis shows the induction of a series of tyrosine phosphorylated species characteristic of Abl transformed cells. Six prominent induced species are 36, 62, 85, 210/220 and 300 kDa (indicated by closed triangles, starting from the bottom), which are identical to those seen in other Abl-transformed cells. The autophosphorylated Abl species is indicated by an open triangle. The induction of autophosphorylation and exogenous phosphorylation by the cIV:ER fusion protein requires at least 1 h for a detectable increase and thereafter increases cumulatively, whereas the induction of phosphorylation of these species occurs to near maximal levels within minutes for the ΔXB:ER fusion protein. The appearance of the 210 kDa band was variable. Molecular weight markers are 46.5, 77, 116.5 and 205 kDa.

(absent in transformed cells)] and transforming versions of the Abl protein, which are predominantly cytoplasmic (Van Etten *et al.*, 1989). To examine whether the localizations of the Abl:ER fusion proteins change upon activation, we analyzed these fusion proteins by indirect immunofluorescence using antisera directed either against the Abl SH3 domain or against the ER HBD.

The cIV:ER fusion protein was observed to be mostly nuclear in cells plated in the absence of hormone and allowed to revert as judged by indirect immunofluorescence staining with affinity-purified antibodies against the Abl SH3 domain (Figure 6A). The majority of staining was due to determinants from the highly overexpressed cIV:ER fusion protein and little staining from endogenous c-Abl as suggested by the much lower level staining of parental NIH 3T3 cells (Figure 6B). A similar pattern of nuclear staining was seen with serum directed against the ER HBD, which showed low background staining of NIH 3T3 cells (Figure 6C and D). We saw no actin association, as would be expected from the fact that the cIV:ER fusion protein lacks the C-terminal actin binding domain.

We examined the localization of the cIV:ER fusion protein at various times after hormone addition and found that over 2–24 h the protein appeared to have a strong nuclear component of anti-Abl SH3 staining, but significant cytoplasmic staining became evident. A representative micrograph showing cIV:ER cells 8 h after hormone addition shows predominantly nuclear staining in some cells and very

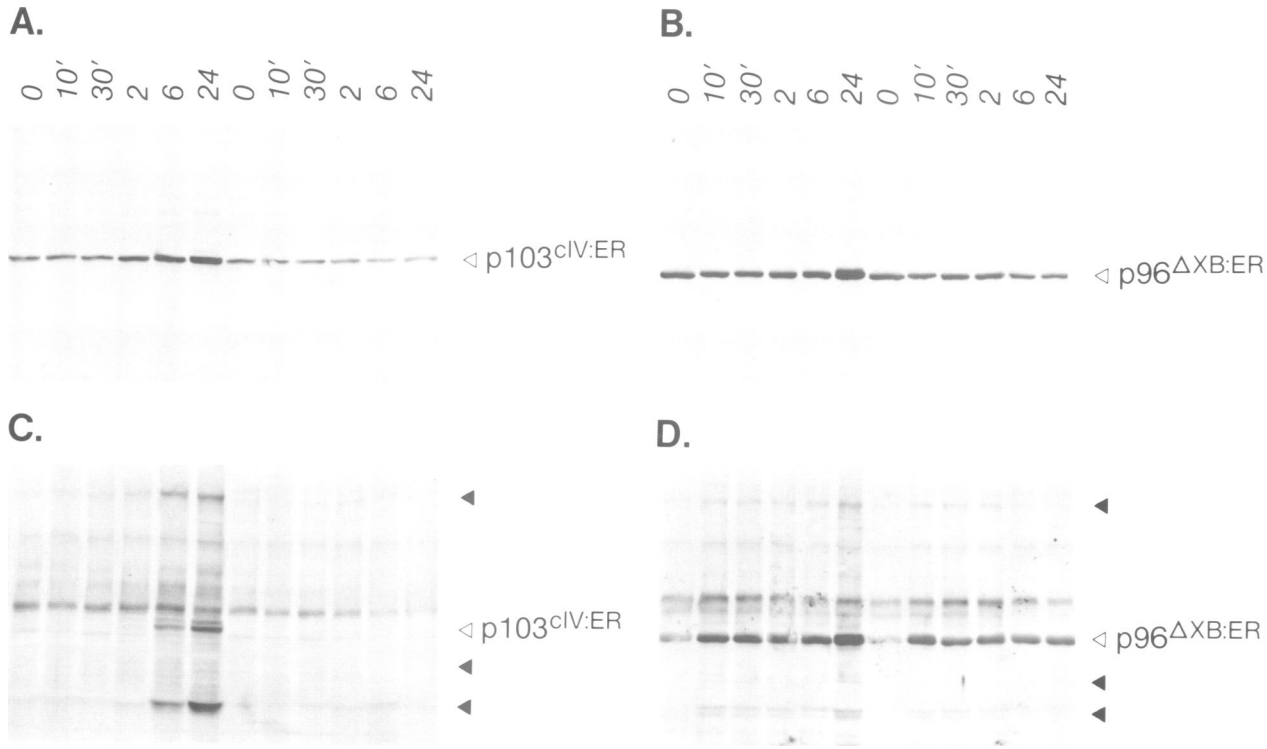

Fig. 5. Activation of the cIV:ER fusion protein kinase requires protein synthesis, whereas the SH3-deleted variant apparently loses this requirement. Clonal lines expressing the cIV:ER and ΔXB:ER fusion proteins were washed, plated without estradiol and grown for 2 days to allow the cells to revert. Multiple plates of reverted cells were treated with hormone and with or without cycloheximide at 100 μg/ml. Extracts were prepared in the presence of the phosphatase inhibitor sodium orthovanadate at the indicated times. Extracts were run on 10% SDS−polyacrylamide gels and analyzed by anti-Abl and anti-phosphotyrosine Western blotting. The four panels are similar to those in Figure 4. In each case, the time course on the left is performed without cycloheximide, and that on the right with cycloheximide. The three closed triangles in panels C and D indicate 62, 85 and 220 kDa proteins; the open triangles indicate the Abl protein.

strong overall staining in others (Figure 6E). In general, the intensity of both nuclear and cytoplasmic staining increased during this time frame. However, between 24 and 48 h after hormone addition, as well as in cells grown continuously in hormone, much of the nuclear staining was lost in most cells, and predominantly cytoplasmic staining was seen (Figure 6F). In cIV:ER cells blocked with cycloheximide and treated with hormone, the protein appeared to remain nuclear, consistent with the cytoplasmic accumulation being due to *de novo* synthesis (data not shown). Thus, the redistribution of the cIV:ER protein concurrent with activation recapitulated the previously reported difference in subcellular localization of c-Abl and transforming versions of Abl (Van Etten *et al.*, 1989; Dhut *et al.*, 1991). However, the kinetics of the redistribution process did not suggest that the protein physically shuttled from the nucleus to the cytoplasm. Rather, the redistribution appeared consistent with a gradual cytoplasmic accumulation of *de novo* synthesized Abl because of an increasing inability of this protein to enter the nucleus in the cells undergoing transformation.

The ΔXB:ER fusion protein remains cytoplasmic

Cells expressing the ΔXB:ER fusion protein showed a distinctive perinuclear staining pattern in the absence of hormone (Figure 6K). Such a subcellular distribution has not been observed for any previously described Abl variant. A kinetic analysis of subcellular localization showed that the ΔXB:ER protein becomes diffusely cytoplasmic lacking the strong perinuclear staining by 24 h after hormone addition (Figure 6L). Throughout the time course, there was no appreciable nuclear staining (data not shown).

Hormone-dependent induction of membrane-associated phosphotyrosine staining

We examined the subcellular localization of the accumulating phosphotyrosine-containing proteins by indirect immunofluorescence. In the absence of hormone, the cells expressing the cIV:ER fusion showed anti-phosphotyrosine antibody staining of punctate and wedge-shaped structures on the ventral surface of the cell (Figure 6G). A similar pattern is seen in parental NIH 3T3 cells and the staining of these structures is blocked with free phosphotyrosine (data not shown). These phosphotyrosine-containing structures have previously been identified as closely associated with focal adhesions and integrin receptors (Tapley *et al.*, 1989; Guan *et al.*, 1991). As early as 2 h after hormone addition, novel focal structures near the ruffling edge of the cell (Figure 6H) and actin-rich cellular processes extending from the plasma membrane (Figure 6I) frequently stain with anti-phosphotyrosine antibody in many cells. Similar membrane structures stain with the anti-Abl SH3 antibodies consistent with the c-Abl:ER fusion being one of the major tyrosine phosphorylated species (data not shown). Highly transformed cells are strongly stained with anti-phosphotyrosine antibodies at 24−48 h after hormone addition (Figure 6J), or in cells maintained in hormone continuously (not shown). A variety of other cytoplasmic structures and a punctate

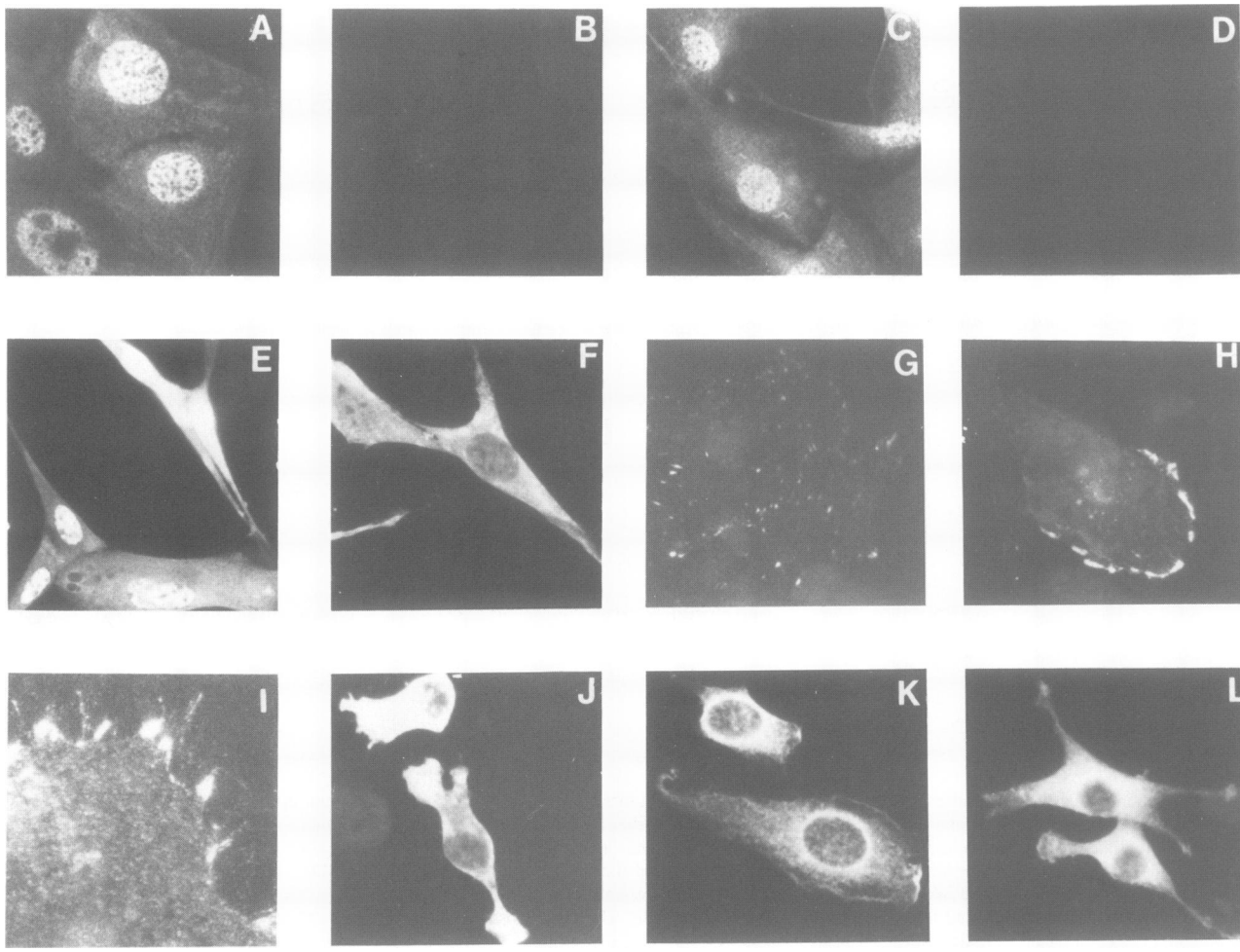

Fig. 6. Hormone-dependent activation of the cIV:ER fusion protein results in a change of localization from nucleus to cytoplasm, while the SH3-deleted variant never localizes to the nucleus. NIH 3T3 lines expressing the cIV:ER and ΔXB:ER fusions were plated in the absence of hormone and allowed to revert for 2 days. The cells were then treated with hormone and fixed for indirect immunofluorescence using methanol−acetone fixation as described in Materials and methods. Primary antibodies were either affinity-purified rabbit antiserum directed against the Abl SH3 domain (A, B, E and F), or rabbit antisera directed against the human ER HBD (C, D, K and L) or the mouse monoclonal antibody 4G10 against phosphotyrosine (G, H, I and J). Secondary antibodies were rhodamine-conjugated donkey anti-rabbit (A−D, K and L) or anti-mouse (G−J). (**A**) cIV:ER-expressing NIH 3T3 cells in the absence of hormone stained with SH3-specific primary serum (800× magnification) showing strong nuclear localization of the cIV:ER fusion protein. (**B**) Parental NIH 3T3 cells stained with SH3-specific primary sera showing low background. (**C**) cIV:ER-expressing NIH 3T3 cells in the absence of hormone stained with ER HBD-specific primary serum confirming the nuclear staining in A. (**D**) Parental NIH 3T3 cells stained with ER HBD-specific primary serum showing low background. (**E**) cIV:ER-expressing NIH 3T3 cells 8 h after hormone addition stained with SH3-specific primary serum (500×) retain nuclear staining, but accumulate cytoplasmic staining. (**F**) cIV:ER-expressing NIH 3T3 cells 48 h after hormone addition stained with SH3-specific primary serum (625×) showing largely cytoplasmic localization. (**G**) cIV:ER-expressing NIH 3T3 cells in the absence of hormone, stained with an anti-phosphotyrosine primary antibody. The focus is on the ventral surface of the cell to emphasize the staining of focal adhesions (500×). (**H** and **I**) cIV:ER-expressing NIH 3T3 cells 8 h after the addition of hormone, stained with an anti-phosphotyrosine primary antibody showing the appearance of membrane phosphotyrosine staining. (H) The focus is on the ruffling edge of the cell (800×). (I) A detailed view showing anti-phosphotyrosine staining in ruffles and spikes (158×). (**J**) cIV:ER-expressing NIH 3T3 cells 48 h after hormone addition stained with an anti-phosphotyrosine primary antibody (625×) showing a large accumulation of tyrosine-phosphorylated proteins. (**K**) ΔXB:ER-expressing NIH 3T3 cells in the absence of hormone stained with ER HBD-specific primary serum (800× magnification) showing a strong perinuclear localization. (**L**) ΔXB:ER-expressing NIH 3T3 cells 48 h after addition of hormone, stained with ER HBD-specific primary serum showing diffuse cytoplasmic localization.

nuclear structure stain with anti-phosphotyrosine antibodies at different times after hormone addition (data not shown). These may represent intermediates in the process of morphological transformation, but a more systematic analysis will be required to establish this idea. Results with ΔXB:ER were essentially similar (data not shown).

Discussion

We have developed a new strategy for constructing regulable derivatives of both normal and activated Abl tyrosine kinases by fusing them to the ER HBD. We have exploited these hormone-conditional Abl:ER fusion proteins to study the kinetics of activation of the Abl kinase activity, the Abl-dependent induction of the transformed state and its influence on redistribution of the Abl protein from the nucleus to the cytoplasm, and to identify a protein synthesis requirement for activation of the Abl kinase.

Fusion to steroid binding domains has previously been used to construct regulable derivatives of several transcription factors. In those cases, the heterologous activity becomes inactivated in a hormone-reversible fashion (Picard

et al., 1988; Eilers *et al.*, 1989; Burk and Klempnauer, 1991; Superti-Furga *et al.*, 1991; Umek *et al.*, 1991). We have now extended the list of the regulable activities to a tyrosine kinase, a transforming variant of c-Abl. Completely unexpected was the finding that a non-transforming, normal c-Abl derivative could be activated for transformation by fusion to the ER HBD. This finding has implications both technically and for the understanding of Abl activation (see below). The activation of c-Abl is also unusual because C-terminal mutations or fusions have not generally been found to activate c-Abl. Further, activation by the ER HBD is relatively specific because C-terminal fusion of other proteins including Gag, Bcr and the ecdysone receptor HBD did not activate c-Abl (P.Jackson and D.Picard, unpublished data).

Hormone-dependent transformation by these fusion proteins was homogeneously conditional and reversible within 24–48 h. We used the regulable Abl:ER protein to study the kinetics of activation of Abl, and found a rapid (~10 min) or slower (over several hours) activation of Abl kinase activity as judged by phosphorylation of cellular substrates, depending on the absence or presence of the SH3 domain, respectively. We correlated the hormone-dependent activation of Abl with increased autophosphorylation, cytoplasmic localization, and the appearance of phosphotyrosine-containing structures at the plasma membrane.

Recently, we observed hormone-dependent formation of a 600–800 kDa complex containing Abl and several tyrosine phosphorylated proteins including p62 and p85 in sucrose gradients, which may correspond to these plasma membrane structures (P.Jackson, D.Baltimore, and D.Picard, unpublished results). For one identified substrate of activated Abl, the 85 kDa subunit of the phosphatidyl inositol-3-kinase (PI-3-kinase), membrane association has been demonstrated to be important for activation of the associated PI-3-kinase activity (Varticovski *et al.*, 1991). We have also observed hormone-dependent coprecipitation of tyrosine phosphorylated proteins including p62, as observed for other transforming Abl proteins (Lugo *et al.*, 1990; Mayer *et al.*, 1992). Here, the Abl SH2 domain could promote complex formation by high affinity binding to tyrosine phosphorylated proteins, a function which appears to be essential for Abl transforming ability (Mayer *et al.*, 1990, 1992).

The c-Abl (IV):ER fusion protein localizes to the nucleus before activation with hormone, whereas upon hormone addition the *de novo* synthesized protein accumulates in the cytoplasm. This result is consistent with our observation that nuclear transport is the default condition for Abl in normal cells and that retention in the cytoplasm is regulated by the transformed state of the cells. In support of this idea, we have recently demonstrated that inducing Abl-dependent transformation can cause an epitope-tagged, nuclear, non-transforming variant of Abl to become retained in the cytoplasm (P.Jackson, D.Baltimore and R.Van Etten, unpublished).

The SH3-deleted Abl:ER fusion protein, ΔXB:ER, never goes to the nucleus and is more rapidly activated, consistent with immediate activation of the kinase without *de novo* synthesis. The failure of the SH3-deleted ΔXB:ER protein to translocate to the nucleus hints at a role for SH3 in modulating nuclear translocation. However, the unique perinuclear localization of and the presence of residual phosphotyrosine within the pool of ΔXB:ER protein in the absence of hormone suggest that this configuration may be a special case, and not generally representative of normal or transforming Abl.

Different mechanisms for inactivation and activation of the Abl kinase?

Dominant inactivation by HBDs has been proposed to be due to the formation of a protein complex involving the heat-shock protein HSP90 and the protein fused to the HBD (for discussion see Picard *et al.*, 1988, 1990; Yamamoto *et al.*, 1988; and references therein). In the absence of hormone, HSP90, which is known to be bound hormone-reversibly to the HBD of steroid receptors, could interfere with various activities of the fusion polypeptide. Upon hormone addition, the release of HSP90 would relieve this inhibition. We suppose that the activated Abl kinase moiety of ΔXB:ER cannot function in the absence of hormone because HSP90, when bound to the HBD moiety, blocks access to substrate proteins or blocks another function essential for Abl-dependent fibroblast transformation, such as myristoylation-dependent membrane association or the SH2-phosphotyrosine binding function (Mayer *et al.*, 1990, 1992; Daley *et al.*, 1992). The fast kinetics (<10 min) and the lack of a protein synthesis requirement for activation of the ΔXB:ER fusion protein is consistent with a rapid release of HSP90 from such complexes.

In cIV:ER, the activation of the non-transforming Abl moiety by the HBD must involve a different mechanism as suggested by several observations: the higher concentrations of hormone required for activation, the different kinetics of morphological change, and the slower kinetics and the protein synthesis requirement for activation of the cIV:ER kinase. Since cIV:ER differs from ΔXB:ER only by the presence of SH3, this kinetic lag suggests that a distinct mechanistic step is required to overcome SH3-dependent negative regulation of Abl activity. The lag also correlates with and may be mechanistically related to the redistribution from the nucleus to the cytoplasm. We do not know whether there are differences in how HSP90 interacts with nuclear cIV:ER versus cytoplasmic ΔXB:ER fusion proteins and whether this explains differences in affinity for estradiol or the dose–response to estradiol for transformation.

Activation of Abl by fusion to proteins promoting oligomerization or by overexpression?

Two hypotheses for the activation of c-Abl by fusion to the ER HBD should be considered: (i) oligomerization and (ii) overexpression.

Oligomerization. The suggested ability of the HBD to promote hormone-dependent dimerization would provide a plausible mechanism for activation (Kumar and Chambon, 1988; Fawell *et al.*, 1990). Since transmembrane tyrosine kinases can be activated by oligomerization, non-receptor tyrosine kinases could be similarly regulated. This mechanism has indeed been suggested for activation of one such kinase, called c-Lck, in T-cell signaling (Bolen and Veillette, 1989). There is no direct biochemical evidence for dimerization/oligomerization of Abl, but others have observed activated versions of Abl in higher order complexes (Li *et al.*, 1988; Campbell *et al.*, 1990; Lugo *et al.*, 1990). The formation of a high molecular weight complex upon hormone addition has so far confounded our attempts to look directly for a hormone-dependent Abl dimer. However, the

possibility of Abl dimerization is supported by the ability of a kinase-active variant of Abl to transphosphorylate a kinase-defective variant in an *in vitro* kinase assay (Witte *et al.*, 1980). Further, when both variants are coexpressed in fibroblasts, the kinase active Abl can transphosphorylate the kinase inactive variant as assayed by anti-phosphotyrosine antibody (P.Jackson and R.Van Etten, unpublished data). An implication of the dimer hypothesis is that the fusion of sequences that promote oligomerization to the Abl protein should activate the Abl kinase activity. The ability of Gag and Bcr sequences found in naturally occurring transforming versions of Abl to dimerize or self-associate is consistent with this prediction (Yoshinaka *et al.*, 1984; Li *et al.*, 1988; Campbell *et al.*, 1990).

Overexpression. The ability of the wild type c-Abl (IV) kinase to be activated *in vivo* by vast overexpression has been demonstrated (Wang, 1988; Lugo *et al.*, 1990; Pendergast *et al.*, 1991). Typically, activation by overexpression has been seen only in transient expression systems and not in stably transfected murine fibroblast lines where such high levels of c-Abl overexpression are precluded by a 'toxic' or cytostatic effect of the c-Abl kinase. We speculate that the toxic effect of the Abl protein has been at least partially suppressed in the Abl:ER fusions, thus allowing overexpression to high levels. This idea is supported by our observation that the Abl:ER fusions are also conditional for the cytostatic effect of the Abl kinase (P.Jackson, D.Baltimore and D.Picard, in preparation). This property of the ER HBD to allow overexpression of 'toxic' proteins may be broadly useful.

Nonetheless, the $p103^{cIV:ER}$ protein expressed in transformed NIH 3T3 cells was not more abundant than the cIV-Δ630 protein expressed in several non-transformed clonal lines (P.Jackson, unpublished data), suggesting that overexpression is not sufficient for transformation, and supporting the notion that the ER HBD has a dominant property causing Abl activation.

Application of regulation by HBDs

The extension of regulation-by-fusion to an HBD from transcription factors to kinases suggests that this strategy may provide regulable alleles for a variety of regulatory and enzymatic moieties, including gene products of unknown biochemical function. Indeed, other tyrosine and serine kinases have now been shown to be regulated as ER fusions (M.McMahon, personal communication). We have recently shown that the kinase activity of the Abl:ER fusion protein is also regulable in *Saccharomyces cerevisiae*, suggesting that the strategy may be generally applicable in eukaryotes (D.Picard and P.Jackson, unpublished). Steroid regulation may also be of considerable use in transgenic animals, especially in mammals, where homeostatic mechanisms generally preclude the use of temperature-sensitive mutants.

Materials and methods

Constructions

Constructs pPLcIV and pPLΔXB, encoding wild type or SH3-deleted versions of c-Abl (IV), respectively, have been described (Jackson and Baltimore, 1989). All new plasmids are based on these retroviral expression vectors.

pPLcIV:ER. A fragment encoding the HBD (amino acids 282–595) of the human ER, originally derived from plasmid HE14 (Kumar *et al.*, 1986), was fused to c-Abl (IV) (amino acids 1–630) at the *Nar*I site following the sequence encoding the pentalysine nuclear localization signal. The HBD used in this study carries a point mutation (Gly to Val at amino acid 400), which lowers ligand affinity (Tora *et al.*, 1989). It is therefore less sensitive to spurious agonist activities in tissue culture medium.

pPLΔXB:ER. Identical to pPLcIV:ER except for the ΔXB deletion of the SH3 domain (Jackson and Baltimore, 1989).

pPLcIV-ΔN. A C-terminal deletion mutant of c-Abl (IV), derived from pPLcIV by inserting a *Spe*I linker in the unique *Nar*I site, thus adding a nonsense mutation 3′ to codon 630. This mutant retains the pentalysine nuclear localization signal.

pPLΔXB-ΔN. Identical to pPLcIV-ΔN, except for the ΔXB deletion of the SH3 domain.

pPLcIV(980):ER. The fragment encoding the HBD of ER was inserted into the unique *Sal*I site of pPLcIV thus fusing c-Abl (IV) amino acids 1–980 to the HBD.

Cell culture and transfection

Transfection of NIH 3T3 cells and generation of clonal cell lines was as described by Jackson and Baltimore (1989). Briefly, 8×10^5 NIH 3T3 cells were cotransfected with 10 μg of plasmid DNA and 0.5 μg of a Moloney virus proviral clone, pZAP (Goff *et al.*, 1982), and scored for transformed foci. Populations of transformed cells were dislodged by squirting with medium, cloned to limiting dilution (≤30 positives per 96 well-plate) and screened for homogeneous hormone-dependent morphological transformation. Putative positive clones were expanded and verified by Western blotting, using the monoclonal antibody 19-84 which is specific for the Abl kinase domain (Schiff-Maker *et al.*, 1986). Independent transformed clones of a given mutant had very similar levels of expression. Immunofluorescence with anti-Abl or anti-ER sera was used to verify the homogeneity of the cloned lines.

Antisera, immunofluorescence and microscopy

Polyclonal rabbit serum specific for c-Abl (IV) was kindly given by Dr Owen Witte (UCLA). Polyclonal rabbit serum against the ER HBD was generously provided by Drs Steve Robbins and Mike Bishop (UCSF). Polyclonal rabbit antiserum was also generated against a GST–SH3 fusion protein (Cicchetti *et al.*, 1992). The SH3 serum was preadsorbed with GST protein and then affinity-purified against the GST–SH3 fusion protein. Both the anti-ER and anti-SH3 sera appeared monospecific for the highly overexpressed Abl:ER fusion proteins by Western blot analysis and SH3 staining could be blocked by including soluble SH3 protein. The anti-Abl monoclonal antibody, 19-84 (Schiff-Maker *et al.*, 1986), was affinity-purified on a goat anti-mouse IgG column from hybridoma supernatant (Cappel). The anti-phosphotyrosine monoclonal antibody, 4G10, was obtained commercially (UBI).

Immunofluorescence was performed as described by Van Etten *et al.* (1989). Briefly, cells grown on coverslips were fixed by methanol–acetone at −20°C, rehydrated, blocked in 5% normal donkey serum, stained with rabbit or mouse serum at 2 μg/ml, washed with PBS, stained with rhodamine-conjugated donkey anti-rabbit or mouse antibody (Jackson Immunoresearch) and counterstained with Hoechst 33358 (Sigma) to mark nuclei. Anti-ER HBD antiserum was used at a 1:300 dilution. Coverslips were mounted with Fluoromount G (Fisher), visualized on a Zeiss Axiophot or Nikon Diaphot, and photographed using TMAX 400 film (Kodak). Additional experiments using fixation with 4% paraformaldehyde and permeabilization with 0.5% Triton X-100 were performed to demonstrate that specific staining patterns were not an artefact of the fixation method. Time-lapse video microscopy was performed by growing cells on 25 mm coverslips and observing the cells under phase-contrast with a 40× air objective on a Zeiss ICM 405 with a CCD camera (Hamaguchi) and video recorder.

Hormone binding assay

The whole cell binding assay was as described by Taylor *et al.* (1984). ER HBD concentrations were determined by measuring hormone binding of whole cells in phenol-red free medium containing 0.1% bovine serum albumin (Sigma) and 1 nM [^{3}H]estradiol (NEN) in the presence or absence of 200 nM unlabeled β-estradiol. Affinity measurements were performed by incubating cells with increasing concentrations of radioligand (0–300 nM). Receptor concentrations and binding constants were deduced using a nonlinear iterative curve fitting program (Murlas *et al.*, 1982).

Western blot analysis and immunoprecipitation

Cell extracts were prepared in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 1% Triton, 0.1% SDS, 1% sodium deoxycholate, 1 mM Na_3VO_4, 40 mM NaF, 30 mM sodium pyrophosphate, 1 mM PMSF, 1% aprotinin, 10 μg/ml pepstatin) and clarified at 13 000 *g* for 15 min at 4°C. Lysates were boiled in sample buffer, run on 5–9% gradient SDS–polyacrylamide gels (50 μg protein for a 3 mm × 1 mm gel slot) and transferred to nitrocellulose or Immobilon (Millipore) for 500 mA-h. Filters were washed in Tris-buffered saline with 0.05% Tween 20 (TBST), blocked for 30 min in 1% BSA/TBST and incubated with affinity-purified anti-Abl or anti-phosphotyrosine antibodies at 2 μg/ml in TBST for 1 h. After three washes in TBST, filters were incubated with 0.5 μg/ml alkaline phosphatase (AP) conjugated donkey anti-mouse (Jackson Immunoresearch, Inc.) in TBST for 30 min. After three washes in TBST and one wash in AP buffer (100 mM Tris pH 9.5, 50 mM NaCl, 2.5 mM $MgCl_2$), filters were reacted in AP buffer plus 300 μg/ml nitroblue tetrazolium (NBT; Promega Biotech) and 150 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Promega Biotech). Filters were photographed using a yellow filter (Hoya).

Metabolic labeling and immunoprecipitations were as described by Jackson and Baltimore (1989).

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References

Anderson,S.M., Rands,E., Fincham,V.J. and Wyke,J.A. (1987) *Virology*, **160**, 263–267.

Bolen,J.B. and Veillette,A. (1989) *Trends Biochem. Sci.*, **14**, 404–407.

Burk,O. and Klempnauer,K.-H. (1991) *EMBO J.*, **10**, 3713–3719.

Campbell,M.L., Li,W. and Arlinghaus,R.B. (1990) *Oncogene*, **5**, 773–776.

Cicchetti,P., Mayer,B.J., Thiel,G. and Baltimore,D. (1992) *Science*, **257**, 803–806.

Daley,G.Q., Van Etten,R.A., Jackson,P.J., Bernards,A. and Baltimore,D. (1992) *Mol. Cell. Biol.*, **12**, 1864–1871.

Dhut,S., Chaplin,T. and Young,B.D. (1991) *Oncogene*, **6**, 1459–1464.

Eilers,M., Picard,D., Yamamoto,K.R. and Bishop,J.M. (1989) *Nature*, **340**, 66–68.

Engelman,A. and Rosenberg,N. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 8021–8025.

Fawell,S.E., Lees,J.A., White,R. and Parker,M.G. (1990) *Cell*, **60**, 953–962.

Franz,W.M., Berger,P. and Wang,J.Y.J. (1989) *EMBO J.*, **8**, 137–147.

Goff,S.P., Tabin,C.J., Wang,J.Y.-J., Weinberg,R. and Baltimore,D. (1982) *J. Virol.*, **41**, 271–285.

Guan,J.L., Trevithick,J.E. and Hynes,R.O. (1991) *Cell Regul.*, **2**, 951–964.

Hatakeyama,M., Kono,T., Kobayashi,N., Kawahara,A., Levin,S.D., Perlmutter,R.M. and Taniguchi,T. (1991) *Science*, **252**, 1523–1528.

Jackson,P. and Baltimore,D. (1989) *EMBO J.*, **8**, 449–456.

Kipreos,E.T. and Wang,J.Y.J. (1992) *Science*, **256**, 382–384.

Kipreos,E., Lee,G.J. and Wang,J.Y.J. (1987) *Proc. Natl Acad. Sci. USA*, **84**, 1345–1349.

Kumar,V. and Chambon,P. (1988) *Cell*, **55**, 145–156.

Kumar,V., Green,S., Staub,A. and Chambon,P. (1986) *EMBO J.*, **5**, 2231–2236.

Li,W., Kloetzer,W.S. and Arlinghaus,R.B. (1988) *Oncogene*, **2**, 559–566.

Lugo,T.G., Pendergast,A., Muller,A.J. and Witte,O.N. (1990) *Science*, **247**, 1079–1082.

Mayer,B.J., Jackson,P.K. and Baltimore,D. (1990) *Proc. Natl Acad. Sci. USA*, **88**, 627–631.

Mayer,B.J., Jackson,P.K., Van Etten,R.A. and Baltimore,D. (1992) *Mol. Cell. Biol.*, **12**, 609–618.

Murlas,C., Nadel,J. and Roberts,J. (1982) *J. Appl. Physiol.*, **52**, 1084–1091.

Pendergast,A.M., Muller,A.J., Havlik,M.H., Clark,R., McCormick,F. and Witte,O.N. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 5927–5931.

Picard,D., Salser,S.J. and Yamamoto,K.R. (1988) *Cell*, **54**, 1073–1080.

Picard,D., Kumar,V., Chambon,P. and Yamamoto,K.R. (1990) *Cell Regul.*, **1**, 291–299.

Prywes,R., Foulkes,J.G. and Baltimore,D. (1985) *J. Virol.*, **54**, 114–122.

Schiff-Maker,L., Burns,M.C., Konopka,J.B., Clark,S., Witte,O.N. and Rosenberg,N. (1986) *J. Virol.*, **57**, 1182–1186.

Seedorf,K., Felder,S., Millauer,B., Schlessinger,J. and Ullrich,A. (1991) *J. Biol. Chem.*, **266**, 12424–12431.

Superti-Furga,G., Bergers,G., Picard,D. and Busslinger,M. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 5114–5118.

Tapley,P., Horwitz,A., Buck,C., Duggan,K. and Rohrschneider,L. (1989) *Oncogene*, **4**, 325–333.

Taylor,C.M., Blanchard,B. and Zava,D.T. (1984) *J. Steroid. Biochem.*, **20**, 1083–1088.

Tora,L., Mullick,A., Metzger,D., Ponglikitmongkol,M., Park,I. and Chambon,P. (1989) *EMBO J.*, **8**, 1981–1986.

Ullrich,A. and Schlessinger,J. (1990) *Cell*, **61**, 203–212.

Umek,R.M., Friedman,A.D. and McKnight,S.L. (1991) *Science*, **251**, 288–292.

Van Etten,R.A., Jackson,P. and Baltimore,D. (1989) *Cell*, **58**, 669–678.

Varticovski,L., Daley,G., Jackson,P., Baltimore,D. and Cantley,L. (1991) *Mol. Cell. Biol.*, **11**, 1107–1113.

Wang,J.Y.J. (1988) *Oncogene Res.*, **3**, 293–298.

Witte,O.N., Goff,S., Rosenberg,N. and Baltimore,D. (1980) *Proc. Natl Acad. Sci. USA*, **77**, 4993–4997.

Yamamoto,K. R., Godowski,P. J. and Picard,D. (1988) *Cold Spring Harbor Symp. Quant. Biol.*, **53**, 803–811.

Yan,H., Schlessinger,J. and Chao,M.V. (1991) *Science*, **252**, 561–563.

Yarden,Y. and Ullrich,A. (1988) *Annu. Rev. Biochem.*, **57**, 443–478.

Yoshinaka,Y., Katoh,I. and Luftig,R.B. (1984) *Virology*, **136**, 274–281.

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