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Growth inhibition by Abl requires an interplay of its SH2 and tyrosine kinase domains

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Abstract Overexpression of c-Abl tyrosine kinase can be growth inhibitory in certain fibroblast cell lines. Using a series of conditional chimeras between Abl and Src, we have now further dissected the Abl protein to determine which domains are required for this function. We found that growth inhibition, unlike transformation by oncogenic forms of Abl, is dependent on the presence of the cognate SH2 and tyrosine kinase domains. Since growth inhibition correlates with low tyrosine kinase activity, it may involve highly specific interactions of target proteins with both domains without the processivity of phosphorylation associated with oncogenic Abl.

Key words: c-*abl* Proto-oncogene; Growth inhibition; SH2 domain; Tyrosine kinase; Fusion protein; Estrogen receptor

1. Introduction

The c-*abl* proto-oncogene encodes a nuclear and cytoplasmic non-receptor tyrosine kinase which contains the Src-homology domains SH3, SH2 (phosphotyrosine binding domain) and SH1 (tyrosine kinase domain) [1]. The majority of our knowledge on Abl derives from studies on numerous oncogenic variants. In contrast, the function of the cellular c-Abl is poorly understood, but several recent observations have suggested a role in the regulation of cell proliferation and differentiation. (1) In Drosophila, Abl has been genetically implicated in the regulation of cell–cell interactions [2]. (2) Mammalian c-Abl is associated with the retinoblastoma protein Rb which inhibits its tyrosine kinase activity and, conversely, overexpression of c-Abl can overcome an Rb-induced growth arrest; moreover, Abl tyrosine kinase is activated during the G1-S transition upon release from the phosphorylated form of Rb [3,4]. (3) Overexpression of wild-type c-Abl [5,6], retroviral v-Abl [7–9] as well as various derivatives thereof [6,10,11] can inhibit the proliferation of murine fibroblast cells; this activity appears to depend on p53 and Rb [12,13]. (4) c-Abl may be involved in the cellular response to DNA-damaging agents [14,15] which is associated with a cell cycle arrest.

We have previously demonstrated that regulable Abl-estrogen receptor (Abl:ER) fusion proteins can be used to facilitate the analysis of the growth inhibitory function of Abl [10]. Abl:ER chimeras can be reversibly switched from their transforming to their growth inhibitory mode by removal of estrogens. This system allowed us to show that growth inhibition correlates with low tyrosine kinase activity of hypo-phosphorylated Abl proteins. Moreover, we found that Abl effects a cell cycle arrest by blocking cells early in G1.

Relatively little is known about the structural requirements for growth inhibition. Of the three Src-homology domains, the SH3 domain is dispensable: it is absent from some of our growth inhibitory Abl:ER chimeras [10,11] and from v-Abl [9]. In contrast, point mutations that inactivate the phosphotyrosine binding activity of SH2 or the tyrosine kinase activity abolish both the transforming and the growth inhibitory functions of Abl [6,13]. Furthermore, the nuclear localization signal at amino acids 624–628 and the C-terminal half of c-Abl, comprising residues 631–1142, are not strictly required for growth inhibition [10] although the latter may play an auxilliary or non-specific role in the context of wild-type c-Abl [10,13]. Thus, these results suggested that the SH2 and tyrosine kinase domains are essential. We now present a further analysis of the contributions of the SH2 and tyrosine kinase domains of Abl to growth inhibition. It reveals that the growth inhibitory function of Abl, similar to specific and processive tyrosine phosphorylation in vitro, requires an interplay between the cognate SH2 and tyrosine kinase domains.

2. Materials and methods

2.1. Plasmid constructions

All constructs were derived from the original full-length Abl–Src chimeras of Mayer et al. [16]. Insertion of sequences encoding the estrogen-receptor hormone binding domain (HBD) was as described [17]. The retroviral vector pGDN [16], which was used for expression, differs only insignificantly from the one used in our previous studies [10,17]. The indicated names refer both to the chimeric proteins themselves and the retroviral vectors used for their expression in NIH 3T3 fibroblast cells. Construct G:ER: fusion of the N-terminal 630 amino acids of c-Abl type IV lacking the SH3 domain to the HBD; the protein G:ER is identical to the one encoded by vector pPLΔXB:ER in our previous study [17]. GSC:ER, GS:ER, and GSC–S:ER are derivatives of G:ER in which the tyrosine kinase domain, the SH2 domain and both domains derive from c-Src.

2.2. Cell culture, transfection, transformation and growth inhibition assays

Transfection of NIH 3T3 cells, generation and propagation of clonal cell lines and determination of 'transformation' and 'inhibition of proliferation' was as described previously [10,11,17]. Where indicated β-estradiol was added at a final concentration of 0.1 μM.

2.3. Immunoblot analysis

The preparation of protein extracts and immunoblotting of identical amounts of total protein were essentially done as previously described [17]. The rat monoclonal antibody H222 (Abbott Laboratories) and the mouse monoclonal anti-phosphotyrosine antibody 4G10 (UBI) were used to reveal fusion proteins containing the estrogen-receptor HBD and proteins containing phosphotyrosine, respectively.

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3. Results and discussion

3.1. Experimental strategy

We took advantage of a series of Abl derivatives in which one or both of these domains (SH2 and tyrosine kinase) are replaced by the corresponding domain(s) of c-Src [16]. The transforming potential of these Abl–Src chimeras is activated due to deletion of the regulatory SH3 domain. These chimeras are all able to transform NIH 3T3 fibroblast cells even though the pattern of phosphotyrosinated cellular proteins shows differences from one derivative to another. To study the growth inhibitory activity of these chimeras, we fused them to the HBD of the estrogen receptor (ER). By analogy to our previously described Abl:ER fusion proteins [10,11], we expected the Abl–Src:ER chimeras to display their transforming and growth inhibitory potential in the presence and in the absence of estrogen, respectively.

As depicted in Fig. 1, vectors for four fusion proteins corresponding to all possible permutations of Abl and Src SH2 and kinase domains were constructed. For simplified cross-reference we have based our nomenclature on the original one of Mayer and Baltimore [16] for the Abl–Src chimeras. Thus, the parent retroviral construct G:ER encodes a fusion protein consisting of the N-terminal 630 amino acids of c-Abl, lacking the SH3 domain, fused to the ER HBD. Note that the protein encoded by G:ER is identical to ΔXB:ER described in our previous studies with fusion proteins [10,17]. The replication-defective retroviral vectors encoding the four chimeras were cotransfected into NIH 3T3 cells with a cloned Moloney helper viral DNA as previously described [10,11,17]. Morphologically transformed foci were scored after 10 days. Foci appeared exclusively in the presence of hormone, indicating that the transforming function of all four fusion proteins was subjected to hormonal control by the ER HBD. Moreover, all constructs induced similar numbers of foci per microgram of transfected DNA (≈10–20/μg DNA). The latter results agree qualitatively with those that were obtained with the same set of Abl–Src chimeras containing the whole C-terminal moiety of c-Abl instead of the ER HBD [16].

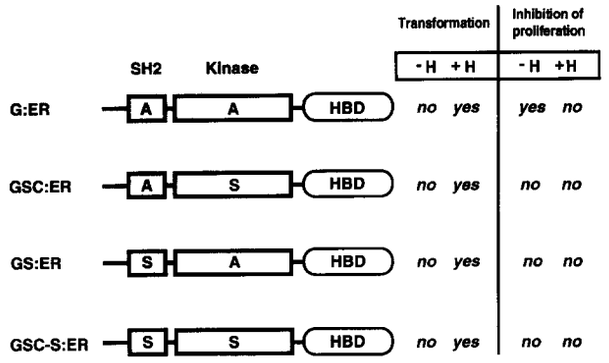

Fig. 1. Schematic representation of Abl derivatives and their biological properties. All four derivatives lack the Abl SH3 domain and are fused to the hormone binding domain (HBD) of the human estrogen receptor at amino acid 630 of murine c-Abl type IV. SH2 and tyrosine kinase domains are boxed; those denoted A and S derive from c-Abl itself and from c-Src, respectively. The column 'Transformation' indicates whether a particular derivative induces morphologically transformed foci in the absence (−H) or in the presence (+H) of β-estradiol. The column 'Inhibition of proliferation' indicates whether overexpression leads to a block of cellular proliferation.

3.2. Morphological transformation correlates with tyrosine kinase activity and is hormone-dependent

Several foci from each transfection were cloned by limiting dilution and maintained in the transformed state in the presence of hormone. When viewed by phase-contrast microscopy (Fig. 2; left panels), cells expressing any of the four chimeras were round and refractile, and grew without apparent contact inhibition. To examine the properties of Abl–Src:ER chimeras in the absence of hormone, cells were washed and incubated in fresh medium without estrogens for 2–3 days. Remarkably, all four types of cell lines reverted to normal morphology upon removal of hormone. This finding is consistent with the hormone-dependence of focus formation which we have observed in the original focus assay.

We have previously shown that hormone-dependent transformation by Abl:ER fusion proteins correlates with hormone-dependent tyrosine kinase activity [17]. We were interested in whether this correlation also applied to the Abl–Src:ER chimeras. Protein extracts were prepared from each cell line grown in the absence and in the presence of hormone and identical amounts of total protein were displayed by SDS-PAGE electrophoresis. Tyrosine-phosphorylated proteins and the fusion proteins themselves were revealed by immunoblotting using antibodies against phosphotyrosine and the ER HBD, respectively (Fig. 3). The four fusion proteins are present at similar levels in the absence or in the presence of hormone although the levels of the chimeras are generally increased by hormone treatment (top panel in Fig. 3). In contrast, for all four fusion proteins, autophosphorylation of the fusion proteins and tyrosine phosphorylation of cellular proteins are dramatically increased upon hormone addition. However, the pattern of cellular proteins, which do become phosphorylated, appears to differ somewhat between different Abl–Src chimeras. In this respect, our fusion proteins with the ER HBD merely recapitulate what had already been observed with the original full-length Abl–Src chimeras [16]. This emphasizes again that there may be different pathways to transformation or that the phosphorylation of very few common cellular targets is sufficient.

The correlation between transformation and tyrosine kinase activity is further strengthened by the behavior of the chimera GSC–S:ER in which both SH2 and tyrosine kinase domain are derived from Src. With this highly artificial protein hormonal control imposed by the ER HBD seems to be somewhat leaky; in the absence of hormone, tyrosine kinase activity is clearly elevated relative to parental NIH 3T3 cells (Fig. 3, bottom panel). This leakiness correlates with a slightly altered cell morphology (Fig. 2) compared to the parental cells, indicating partial transformation.

3.3. Growth inhibition requires both the Abl SH2 and the Abl tyrosine kinase domains

We have thus shown for all four chimeras that the transforming function can be switched off by removal of hormone. In the case of Abl:ER fusion proteins such as G:ER, we knew that this results in the concomitant activation of the growth inhibitory function of Abl. By examining the proliferation of cell lines expressing the Abl–Src:ER chimeras GSC:ER, GS:ER and GSC–S:ER after removal of hormone, we could now assess the role of the SH2 and the tyrosine kinase domains of Abl in growth inhibition. The effects of Abl–Src:ER

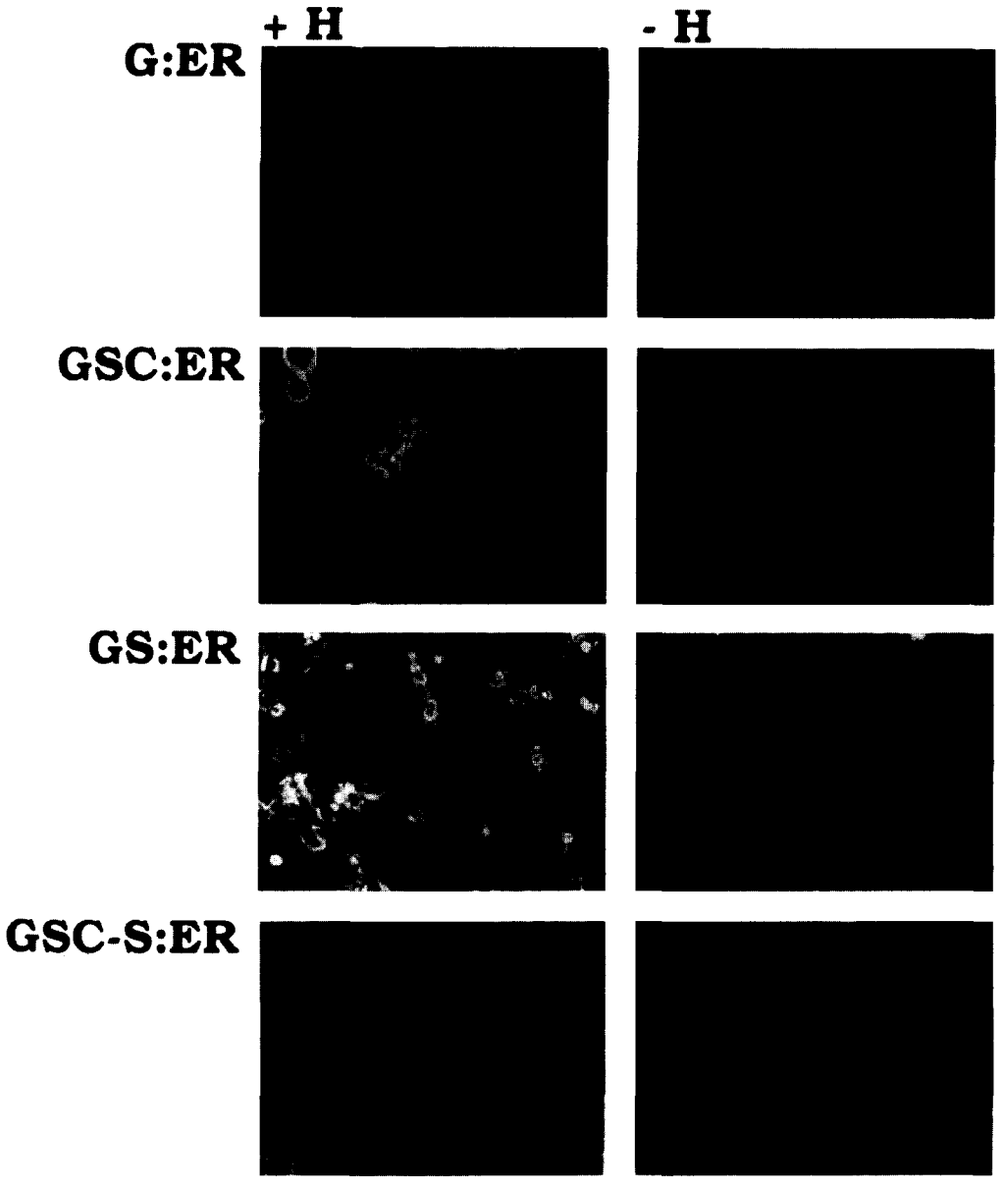

Fig. 2. Reversible morphological transformation of clonal cell lines. Cell lines expressing the Abl:ER fusion protein G:ER and the Abl–Src:ER fusion proteins GSC:ER, GS:ER and GSC–S:ER were grown in the presence (+H) or in the absence (−H) of β-estradiol for 48 h. Phase-contrast micrographs show transformed and normal morphology, as defined by Jackson et al. [17], in the presence and in the absence of hormone, respectively.

chimeras on proliferation were determined by establishing growth curves for clonal cell lines in the absence and in the presence of hormone.

Fig. 4 shows the results for each of the four chimeras. For a given chimera, all clones, which were examined, showed the same behavior. In the presence of hormone, that is in the transformed state, cell lines expressing G:ER and Abl–Src:ER chimeras grow exponentially at similar rates. Subtle differences in growth rates, notably between GS:ER cells and cells expressing other chimeras, may be due to differential tyrosine phosphorylation of cellular target proteins which we have observed by our immunoblotting experiment with anti-phosphotyrosine antibodies (see Fig. 3). Upon removal of hormone, a striking difference between G:ER and the other chimeras is revealed. As predicted, G:ER is switched to its growth inhibitory mode and completely blocks further proliferation. Although G:ER cells could be expected to progress through the cell cycle until they reach the point of G1 arrest, no further increase in G:ER cell numbers can be observed. As we have previously pointed out [10], it is conceivable that a fraction of cells undergoes cell death before the cells arrest in G1. In contrast, cells expressing Abl–Src:ER chimeras GS:ER, GSC:ER or GSC–S:ER continued to proliferate and doubled 3–5 times over a period of 6 days. The growth rates were slightly reduced in this particular set of experiments in the absence of hormone, but in other experiments, the growth rates of GS:ER, GSC:ER and GSC–S:ER cells were similar with and without hormone and comparable to that of the parental NIH 3T3 cells (data not shown). Given this variability, we cannot exclude that Abl–Src:ER chimeras GS:ER, GSC:ER and GSC–S:ER might retain a very low level of growth inhibitory activity. However, the dramatically

different growth properties of cells expressing G:ER or the Abl–Src chimeras strongly suggest that a specific interplay of the Abl SH2 and tyrosine kinase domains is essential for the growth inhibitory activity of Abl.

We have now demonstrated that the growth inhibitory function of Abl requires both its SH2 domain and its tyrosine kinase domain. Mutants, in which one or the other or both domains are replaced by those of Src, lose their ability to inhibit proliferation. This suggests a specific interplay between the two domains. It has recently been shown that tyrosine kinases preferentially phosphorylate substrates recognized by their own SH2 and it was suggested that this increases substrate specificity [18–20]. Indeed, Duyster et al. [18] and Mayer et al. [19] reported that the specificity and the processivity of the Abl tyrosine kinase domain is dependent on the presence of its own SH2 domain. While these studies have established the SH2-dependence of tyrosine kinases in their active state in vitro, we have now extended the notion of interdependence of SH2 and SH1 (tyrosine kinase domain) to the growth inhibitory function of Abl in vivo. Substrate specificity can also be influenced by small adapter proteins such as Crk and Grb2 which contain an SH2 domain and bind to Abl via their SH3 domain [19,21]. Since all our chimeras contain the binding sites for these adapter proteins, our results indicate that it is the SH2 domain of Abl itself which is necessary for growth inhibition.

Interestingly, the growth inhibitory activities of wild-type c-Abl as well as Abl:ER fusion proteins correlate with very low tyrosine kinase activity; nevertheless, a functional tyrosine kinase domain is absolutely required [6,13]. We have previously speculated that growth inhibition may depend on non-catalytic interactions of the tyrosine kinase domain or on low levels of tyrosine phosphorylation of target proteins. The additional requirement for the SH2 domain is compatible with both models. According to the first model, the SH2 domain may help to capture target proteins which have already been phosphorylated, conceivably by other tyrosine kinases; this interaction might facilitate a non-catalytic interaction between the Abl tyrosine kinase domain and the same target protein or even a third protein. An example of such a non-catalytic interaction has indeed been described: the retinoblastoma protein Rb binds the ATP binding pocket of Abl and inhibits the tyrosine kinase activity in a cell-cycle-dependent way [3]. However, whether Rb binding is important for Abl-mediated growth inhibition remains unclear. While the presence of Rb appears to be necessary for growth inhibition by Abl [13], binding alone is clearly not sufficient as exemplified by two types of mutants. Our chimera GS:ER, which contains the Abl tyrosine kinase domain and thus would be expected to bind Rb, fails to be growth inhibitory, and a point mutation in the kinase domain does not affect Rb binding [3] but abolishes growth inhibition [6,13].

The second model predicts that key target proteins would get phosphorylated by Abl despite the low overall kinase activity; the cognate SH2 domain might then bind these proteins and possibly preserve the tyrosine phosphorylation by preventing access of phosphatases. While a functional kinase is a prerequisite for this model, high stoichiometry and processivity of phosphorylation is not. Indeed, proteins which become specifically phosphorylated under growth inhibitory conditions cannot be detected by immunoblotting (see Fig. 3). Both models predict that the interplay of the SH2 and SH1 domains leads to the assembly of a specific complex of key regulatory protein(s) on Abl. Growth inhibition could

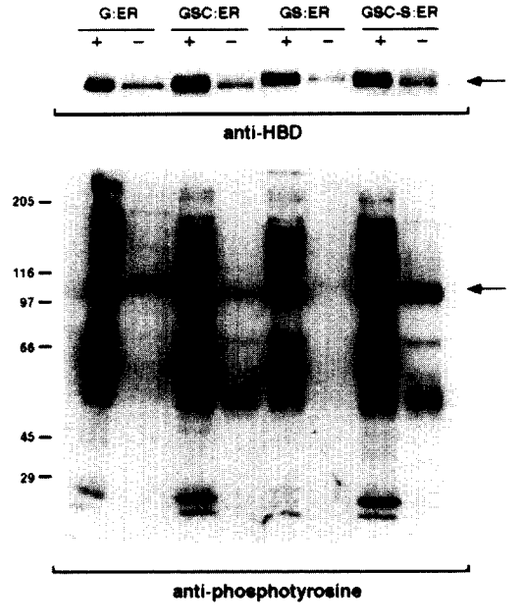

Fig. 3. The tyrosine kinase activities of Abl–Src:ER chimeras are regulated and very low in the absence of hormone. Immunoblot analysis of the chimeric proteins themselves and of total cellular phosphotyrosinated proteins. (Upper panel) The fusion proteins were revealed with an antibody directed against the ER HBD. (Lower panel) Proteins containing phosphotyrosine were revealed on the same blot. Note that Abl chimeras become strongly autophosphorylated in the presence of hormone. The positions of molecular weight markers are indicated on the left. Arrows on the right mark the positions of the Abl chimeras which are all almost identical in size.

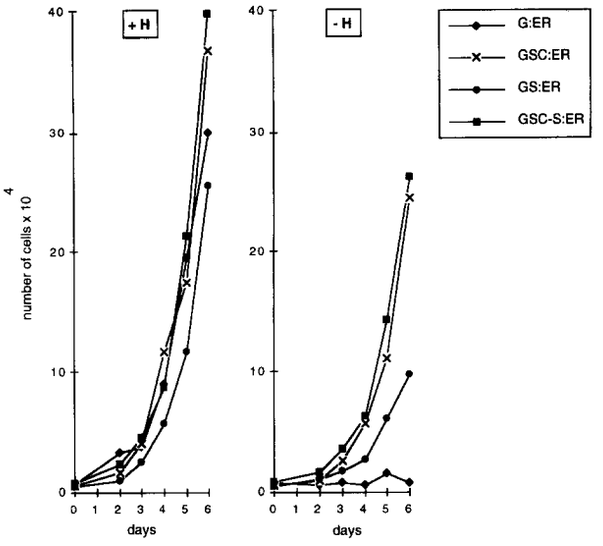

Fig. 4. The substitution of the SH2 or the tyrosine kinase domains abolishes growth inhibition. Growth curves of clonal cell lines expressing Abl:ER and Abl–Src:ER chimeras. Cells were washed and 5000 to 10,000 cells were plated in each well of a 24-well plate, with (left panel +H) or without (right panel −H) β-estradiol; cells were counted at 1 day intervals starting 48 h after plating. The graphs represent the average from two different clones for each of the chimeras. Three independent experiments gave qualitatively identical results.

then result from the sequestration of factors or from the formation of such a complex.

We hypothesize that the growth regulatory function of Abl revealed by our experiments is a reflection of the normal role of the endogenous c-Abl. If this is the case, one would expect that Abl-Src chimeras would also fail to complement knockout mice and flies.

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References

[1] Wang, J.Y.J. (1993) Curr. Opin. Genet. Dev. 3, 35–43.
[2] Bennett, R.L. and Hoffmann, F.M. (1992) Development 116, 953–966.
[3] Welch, P.J. and Wang, J.Y. (1993) Cell 75, 779–790.
[4] Welch, P.J. and Wang, J.Y. (1995) Mol. Cell. Biol. 15, 5542–5551.
[5] Jackson, P. and Baltimore, D. (1989) EMBO J. 8, 449–456.
[6] Sawyers, C.L., McLaughlin, J., Goga, A., Havlik, M. and Witte, O. (1994) Cell 77, 121–131.
[7] Ziegler, S.F., Whitlock, C.A., Goff, S.P., Gifford, A. and Witte, O.N. (1981) Cell 27, 477–486.
[8] Watanabe, S.M. and Witte, O.N. (1983) J. Virol. 45, 1028–1036.
[9] Renshaw, M.W., Kipreos, E.T., Albrecht, M.R. and Wang, J.Y.J. (1992) EMBO J. 11, 3941–3951.
[10] Mattioni, T., Jackson, P.J., Bchini-Hooft van Huijsduijnen, O. and Picard, D. (1995) Oncogene 10, 1325–1333.
[11] Mattioni, T., Louvion, J.-F. and Picard, D. (1994) in: Protein Expression in Animal Cells (Roth, M., Ed.), Methods in Cell Biology, Vol. 43, pp. 335–352, Academic Press.
[12] Goga, A., Liu, X., Hambuch, T.M., Senechal, K., Major, E., Berk, A.J., Witte, O.N. and Sawyers, C.L. (1995) Oncogene 11, 791–799.
[13] Wen, S.-T., Jackson, P.K. and Van Etten, R.A. (1996) EMBO J. 15, 1583–1595.
[14] Kharbanda, S., Ren, R., Pandey, P., Shafman, T.D., Feller, S.M., Weichselbaum, R.R. and Kufe, D.W. (1995) Nature 376, 785–788.
[15] Kharbanda, S., Pandey, P., Ren, R., Mayer, B., Zon, L. and Kufe, D. (1995) J. Biol. Chem. 270, 30278–30281.
[16] Mayer, B.J. and Baltimore, D. (1994) Mol. Cell. Biol. 14, 2883–2894.
[17] Jackson, P., Baltimore, D. and Picard, D. (1993) EMBO J. 12, 2809–2819.
[18] Duyster, J., Baskaran, R. and Wang, J.Y. (1995) Proc. Natl. Acad. Sci. USA 92, 1555–1559.
[19] Mayer, B.J., Hirai, H. and Sakai, R. (1995) Curr. Biol. 5, 296–305.
[20] Songyang, Z. et al. (1995) Nature 373, 536–539.
[21] Tanaka, M., Gupta, R. and Mayer, B.J. (1995) Mol. Cell. Biol. 15, 6829–6837.