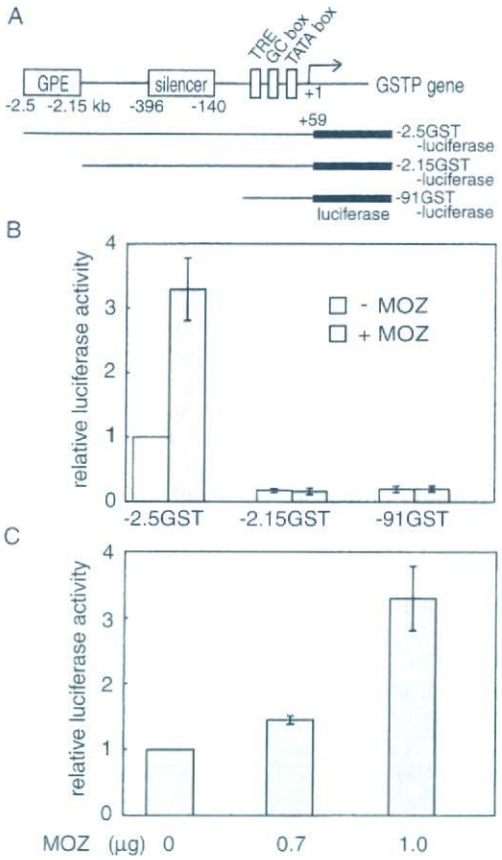

Figure 3 MOZ activates the GSTP promoter activity through the GPE

(**A**) Diagram of the 5′-flanking region of the rat GSTP gene and the reporter constructs for observing the effect of MOZ on the promoter activity of the GSTP gene. (**B**) We co-transfected 100 ng of the reporter plasmid with (grey columns) or without (white columns) 1 μg of MOZ expression plasmid (pCI-MOZ) into H4IIE rat hepatoma cells. All transfection assays were repeated at least three times. The relative luciferase activity was calculated from mean values relative to the activity of −2.5GST-luciferase in the absence of MOZ. Each error bar indicates ±S.D. (**C**) Dose-dependent transactivation of −2.5GST-luciferase by MOZ. Relative luciferase activities are shown as in (**B**).

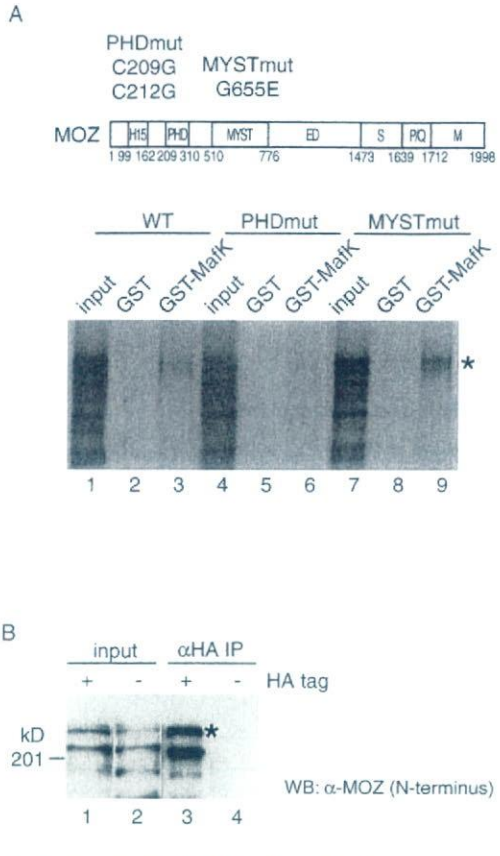

Figure 4 MOZ interacts with MafK *in vitro* and *in vivo*

(**A**) Structural domains of MOZ were indicated as follows: H15, histones H1- and H5-like module; MYST, MYST acetyltransferase domain; ED, glutamic acid/aspartic acid-rich acidic regions; S, serine-rich domain; P/Q, proline/glutamine-stretch; and M, methionine-rich domain. Also shown are the mutation positions in the PHD finger and MYST regions. Indicated wild-type and mutated *in vitro*-translated [^{35}S]MOZ proteins were incubated with GST (lanes 2, 5 and 8) or GST–MafK (lanes 3, 6 and 9). MOZ protein retained on the GST-conjugated beads after extensive washing was analysed by SDS/PAGE and autoradiography. The amount of input (lanes 1, 4 and 7) is equivalent to 10% of the reaction volume in the assay. [^{35}S]MOZ proteins are indicated by asterisks (*). (**B**) MOZ expression plasmid was co-transfected with HA-tagged MafK (lanes 1 and 3) or non-tagged MafK (lanes 2 and 4) into HeLa cells, and nuclear extracts were prepared. Immunoprecipitation (IP) experiments were performed with anti-HA antibody. Immunoprecipitates (lanes 3 and 4) and 5% of input (lanes 1 and 2) were resolved by SDS/PAGE (7.5% gel) and detected by Western blotting using anti-N-terminal MOZ antibody. MOZ proteins are indicated by asterisks (*).

between MOZ and the Nrf2 DNA-binding domain was not detected (results not shown). Next, we evaluated the interaction between MOZ and MafK using a GST pull-down assay and found that ^{35}S-labelled MOZ interacted with GST–MafK but not with GST alone (Figure 4A, lanes 1–3). Unique structural domains are identified in MOZ [28]. To identify the region required for the interaction between MOZ and MafK, two MOZ derivatives with double and single point mutations in the PHD zinc-finger (C209G and C212G) and the MYST (G655E) regions respectively, were generated. The PHD zinc-finger and the MYST regions are important for binding to specific nuclear protein partners and HAT activity, respectively [43,44]. The mutant in the PHD zinc-finger region was not able to interact with GST–MafK, whereas the mutation in the putative acetyl-CoA-binding site in the MYST region did not affect the binding to MafK (Figure 4A, lanes 4–9). These results suggest that MOZ interacts with MafK in the absence of the heterodimer partner, Nrf2, mediated by the PHD zinc-finger region of MOZ.

To evaluate the interaction between MOZ and MafK under physiological conditions, we attempted to detect immunoprecipitated MOZ, but endogenous MOZ in nuclear extracts from H4IIE and HeLa cells could not be detected. Therefore we next introduced the MOZ expression plasmid with HA-tagged or non-tagged MafK into HeLa cells, and nuclear extracts were prepared. MOZ was immunoprecipitated only in nuclear extracts expressing HA-tagged MafK (Figure 4B). Some degraded MOZ proteins were detected in nuclear extracts and these proteins were also immunoprecipitated. GST pull-down and immunoprecipitation experiments suggest that MOZ may interact with the MafK moiety of the Nrf2–MafK heterodimer *in vivo*.

MOZ functions as a co-activator of the Nrf2–MafK heterodimer

MOZ preferentially interacted with MafK and up-regulated GSTP promoter activity through GPE, which contains the binding site for the Nrf2–MafK in the reporter assay (Figures 3 and 4). These data suggest that MOZ is a potential co-activator of Nrf2–MafK heterodimer. To test this hypothesis, we investigated whether MOZ could stimulate Nrf2–MafK-mediated transactivation (Figure 5). We have previously reported that Nrf2 simulates GPE1-mediated transactivation in F9 cells, which are considered to lack AP1 (activator protein 1) activity and to express excess amounts of small Maf proteins, including MafK [34]. MOZ or Nrf2

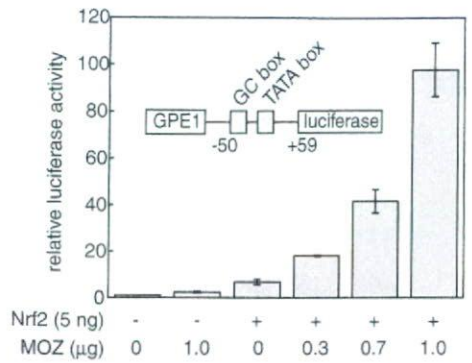

Figure 5 MOZ is a co-activator of Nrf2

Nrf2-mediated transactivation by MOZ was examined in mouse embryonic carcinoma F9 cells. We co-transfected 100 ng of the reporter plasmid (GPE1-luciferase, in the panel) and 5 ng of *Renilla* luciferase plasmid (phRL-tk) with 0, 0.3, 0.7 and 1 μg MOZ expression plasmid (pCI-MOZ) in the absence (–) or presence (+) of Nrf2 expression plasmid (pAβ2-Nrf2, 5 ng). The luciferase activity was normalized to *Renilla* luciferase activity. Relative luciferase activity was calculated from the mean values relative to the activity of GPE1-luciferase without Nrf2 and MOZ. Each error bar indicates $\pm$S.D.

Figure 6 MOZ induces endogenous GSTP expression

H4IIE cells were transfected with 0, 0.4, 0.7 and 1.0 μg of MOZ expression plasmid (pCI-MOZ, lanes 1–4), and cell lysates were prepared. Endogenous GSTP and GAPDH ('G3PDH') were detected by immunoblotting.

expression plasmid was co-transfected with the reporter plasmid GPE1-luciferase (which includes GPE1 and the GSTP promoter) into F9 cells. MOZ and Nrf2 slightly enhanced the activity of the reporter construct. As expected, MOZ, when in the presence of Nrf2, dose-dependently stimulated GPE1-mediated GSTP promoter activity. MOZ did not stimulate the promoter activity of reporter plasmids including the mutated Nrf2 binding site (results not shown).

Induction of endogenous GSTP expression by MOZ in rat hepatoma cells

As we described above, MOZ stimulated the GSTP promoter activity mediated by Nrf2–MafK. We then assessed the effects of MOZ overexpression on GSTP expression in H4IIE cells. Transiently overexpressed MOZ induced expression of endogenous GSTP but not GAPDH (Figure 6). The induction of GSTP protein was dependent on the exogenous MOZ expression. These results suggest that MOZ functions as a co-activator of the Nrf2–MafK heterodimer and may stimulate GSTP gene expression during hepatocarcinogenesis.

DISCUSSION

HATs contribute to tumour suppression, and loss or dysregulation of these activities may be linked to tumorigenesis [45]. To gain insight into the roles of HATs in liver cancer, we analysed the expression profiles of HATs during hepatocarcinogenesis and evaluated their roles in hepatocarcinogenic-specific gene expression.

We have shown that MOZ expression was up-regulated during hepatocarcinogenesis. MOZ functions as a co-activator of AML1-mediated transcription, and the AML1–MOZ complex might play a role in cell differentiation [28,42]. MOZ frequently is rearranged in leukaemia, and the MOZ fusion protein antagonizes MOZ function in haemopoiesis [26,28]. Even though MOZ rearrangement does not occur during hepatocarcinogenesis, we documented an anomalous increase of MOZ. Because HATs regulate global gene expression [1,46], dysregulation of MOZ may induce unusual gene expression, leading to hepatocarcinogenesis.

Recently, the MOZ–MORF complex including BRPF (bromodomain- and PHD finger-containing) 1/2/3 paralogue and ING5 (inhibitor of growth 5; tumour suppressor) was purified [47]. MORF was not detected in nuclear extracts from livers with hyperplastic nodules (results not shown), so that MOZ, but not MORF, complex may regulate GSTP expression. ING5 is also included in HBO1 [HAT binding to ORC1 (origin recognition complex subunit 1)] complex. Interestingly, ING4, another member of ING family proteins, exists in HBO1 complex, but not MOZ complex. AML1-dependent promoter activity is stimulated by ING5, but not ING4 [47]. This raises a possibility that overexpressed MOZ may affect regulation of AML1-dependent gene expression. ING5 tumour suppressor is included in both HBO1 and MOZ complexes, which are important for DNA synthesis [47]. Overexpressed MOZ might trap ING5 and generate partial complexes, and further, HBO1 complex would be affected with the change of ING5 level. Thus aberrantly expressed MOZ during hepatocarcinogenesis may disturb the tumour suppressor function of ING5 complexes and DNA synthesis, which lead to tumorigenesis.

We also found that the expression of p300 and CBP were decreased during hepatocarcinogenesis. Although AAF blocks the proliferation of hepatocytes, GSTP-expressing cells escape from the growth inhibition and continuously grow in the Solt–Farber model. Trautwein et al. [48] reported that AAF blocks cell-cycle progression after PH by inducing the cyclin-dependent kinase inhibitor p21. Expression of p21 is regulated mainly by the tumour-suppressor protein p53, and full transcriptional activity of p53 requires the co-activators p300/CBP [49–51]. Down-regulation of p300 and CBP reduces p53 activity and leads to cell-cycle progression of GSTP-expressing cells, suggesting that p300 and CBP may be considered tumour suppressors, and their loss of function may be a link to hepatocarcinogenesis.

GSTP is a Phase II detoxification enzyme involved in the metabolism of carcinogens, and it plays a protective role during chemical hepatocarcinogenesis [52]. The Nrf2–MafK heterodimer is important for the GSTP expression during early hepatocarcinogenesis, but it is difficult to explain the markedly increased expression of GSTP in livers with hyperplastic nodules solely on the basis of the increased quantity of Nrf2 [34]. We found that the expression of MOZ was well correlated with GSTP expression during hepatocarcinogenesis; MOZ also functioned as a co-activator of the Nrf2–MafK heterodimer. We reported that the binding activity of Nrf2–MafK heterodimer to GPE1 is much stronger than that of MafK homodimer. Further Nrf2 alone could not bind to GPE1, and the Nrf2 mRNA level is increased in cells from hyperplasic nodules when compared with those from normal livers [34]. Histones H3 and H4 are acetylated in both GPE1 and in the promoter regions of the GSTP gene in the H4IIE hepatoma cell line but not normal liver [34]. This acetylation coincides with the activation of GSTP expression. MOZ may contribute acetylation of histones in the regulatory region of the GSTP gene. Elevation of both MOZ and Nrf2 expression may be required for the dramatically increased gene expression of GSTP observed during hepatocarcinogenesis *in vivo*. To understand

the molecular mechanism of the GSTP induction mediated by Nrf2–MafK heterodimer and MOZ, we proceeded to identify the regions of MOZ and MafK required for the GSTP expression in exogenously Nrf2-expressed H4IIE cells.

The activation mechanism of GSTP expression is classified into two types: specific induction in livers with hyperplastic nodules by chemical carcinogens, and non-specific induction by non-carcinogenic agents such as antioxidants [29,53]. The former induction may require both Nrf2 and MOZ, but only Nrf2 may be necessary for the latter. Preneoplastic foci and nodules are derived from GSTP-positive single cells [54]. The mechanism of the generation of the GSTP-positive single cell is unclear, and specific induction of GSTP has not been reproduced in cell lines by using chemical carcinogens. The use of transgenic or MOZ knockout animals would probably enable us to demonstrate the mechanism of chemical carcinogen-associated GSTP induction during hepatocarcinogenesis.

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〈論 文—Original Paper〉

PRTR化学物質の各種核内受容体に対する結合性

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Binding Affinity of PRTR Chemicals to Various Human Nuclear Receptors

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Abstract

Since the 1990s, population decreases, reproductive anomalies and malformations of highly aquatic animals have been increasingly reported. One possible cause is considered to be endocrine disruptive effects induced by environmental contaminants through a direct interaction with nuclear receptors, not only with steroid hormone receptors but also with other ones. In this study, we examined the binding affinities of 20 chemicals, which are registered in the Japanese Pollutant Release and Transfer Register (PRTR) and have been abundantly discharged into aquatic environments to eight human nuclear receptors and assessed their potential endocrine disruptive effects. Of the 20 PRTR chemicals tested, nonylphenol diethoxylate, telephthalic acid (TPA), and linear dodecyl-benzensulfonate (DBS) bound to at least two receptors at high concentrations. TPA and DBS enhanced the activities of both retinoic acid receptor (RAR) γ and vitamin D receptor (VDR) in a dose-dependent manner. This suggests that TPA and DBS may disturb the vitamin D endocrine functions mediated by a VDR-VDR homodimer or a VDR-RAR heterodimer. Also, our results indicate that endocrine disruptors unsuspected under the current assessment criteria could potentially bind to various nuclear receptors and disrupt endocrine systems mediated by such receptors.

Key words: aquatic environment, endocrine disruptive effect, nuclear receptor, PRTR chemical

1. はじめに

1990年代から，魚類や両生類など，水への依存度の高い野生生物種において，個体数の減少，生殖異常，形態異常の発生が数多く報告されている[1-6]。このような危機的状況をもたらした原因の一つは，環境中に放出された人工化学物質のホルモン様作用に起因する正常な内分泌バランスの攪乱にあるとされている[7]。内分泌攪乱化学物質（endocrine disruptors ; EDs）の内分泌機能への影響発現には，核内受容体（nuclear receptor ; NR）を介するメカニズムと介さないメカニズムが存在するが，その大部分はNRへの直接作用によると考えられている[8]。NRの中には，ヒトを含め，異なる生物種間で高い保存性を示すものもあることから[9]，EDsによる内分泌機能攪乱に伴う悪影響

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がヒトにも生じる可能性があるものと推測される。

従来，EDsに関する研究は，主にエストロゲン受容体（estrogen receptor；ER）などの性ホルモン受容体や甲状腺ホルモン受容体（thyroid hormone receptor；TR）を中心に進められてきた。しかし近年，ヒトのNRファミリーに48種類の受容体が存在することが断定され[10]，EDsの作用点が性ホルモン受容体やTR以外のNRにもある可能性が議論されるようになった[11,12]。例えば，プラスチックの可塑剤等に使用されるフタル酸ジエステルの生体内代謝物であるフタル酸モノエステルによる雌生殖毒性等の生態毒性にはペルオキソーム増殖剤活性化受容体（peroxisome proliferator-activated receptor；PPAR）[13,14]が，また有機スズ化合物によるイボニシ貝のインポセックス発達の促進にはレチノイドX受容体（retinoid X receptor；RXR）[15]が関与していることが示唆されている。また，地下水汚染物質の一つ，トリクロロエチレンの生体内代謝物であるトリクロロ酢酸とジクロロ酢酸による肝臓癌とPPARαの関連性も指摘されている[16,17]。これらの新たな科学的事実から，化学物質の内分泌攪乱活性を評価するためには，様々なNRに対する作用を網羅的に検討することが重要であるといえる。すなわち，これまでに性ホルモン受容体やTRについて内分泌攪乱作用がないと判定された化学物質を含め，多くの人工化学物質のNRに対する作用を検討していくことが必要である。本研究では，水環境中に大量に放出されている化学物質のヒトNRに対する結合性を調査し，潜在的な内分泌攪乱作用の可能性を探った。被検物質には，環境汚染物質排出移動登録（pollutant release and transfer register；PRTR）制度の第1種指定化学物質に含まれ，公共用水域への年排出量が15tを上回る有機化学物質群の中から20種類を選定した。被検物質のNRに対する結合性の評価は，ヒトNRのうちERα，TRα，ビタミンA受容体（retinoic acid receptor；RAR）γ，RXRα，ビタミンD受容体（vitamin D receptor；VDR），PPARα/γ/δを対象として，*in vitro*でNRとコアクチベーターの相互作用を検出できるハイスループットスクリーニング法であるCoA-BAP（coactivator-bacterial alkaline phosphatase）法[18]を用いて実施した。

2．方　　法

2.1　化学物質

標準リガンドとして，ERαには17β-エストラジオール（E2），TRαには3,3',5-トリイオド-L-チロニン（T3），RARγには*all-trans*-レチノイン酸（retinoic acid；RA），RXRαには9-*cis* RA，VDRには1α,25-ジヒドロキシビタミンD3（$1,25(OH)_2D3$），PPARαにはGW7647，PPARγにはRosiglitazone，PPARδにはGW501516を用いた。

PRTR制度で第1種指定化学物質に指定され，公共用水域への年間排出量15t以上で，水より蒸気圧が低い有機化学物質群の中から20種類の化学物質（**Table 1**）を被検物質として選定した。直鎖ドデシルベンゼンスルホン酸ナトリウム（linear dodecyl-benzenesulfonate；DBS）は，直鎖アルキルベンゼンスルホン酸塩（linear-alkylbenzenesulfonate；LAS）の代表として選出した。また，ノニルフェノールジエトキシレート（nonylphenol diethoxylate；NP2EO）は，ノニルフェノールポリエトキシレート（nonylphenol polyethoxylate；

Table 1　Binding affinity of 20 PRTR chemicals for various nuclear receptors[a]

No.	Compound	ERα	TRα	RARγ	RXRα	VDR	PPARα	PPARγ	PPARδ
1	Ethylene glycol	−	−	−	−	−	−	−	−
2	N,N-Dimethylformamide	−	−	−	−	−	−	−	−
3	Nonylphenol diethoxylate (NP2EO)	++	−	−	−	−	−	+	−
4	Thiourea	−	−	−	−	−	−	−	−
5	ε-Caprolactam	−	−	−	−	−	−	−	−
6	Ethylenediaminetetraacetic acid	−	−	−	−	−	−	−	−
7	Terephthalic acid (TPA)	−	−	++	−	++	−	+	−
8	Diethylenetriamine	−	−	−	−	−	−	−	−
9	Toluene	−	−	−	−	−	−	−	−
10	1,4-Dioxane	−	−	−	−	−	−	−	−
11	Methacrylic acid	−	−	−	−	−	−	−	−
12	2-Aminoethanol	−	−	−	−	−	−	−	−
13	Acrylic acid	−	−	−	−	−	−	−	−
14	Linear dodecyl-benzenesulfonate (DBS)	−	+	++	−	++	−	+	−
15	1,3-Dichloro-2-propanol	−	−	−	−	−	−	−	−
16	Hexamethylenediamine	−	−	−	−	−	−	−	−
17	*p*-Xylene	−	−	−	−	−	−	−	−
18	Aniline	−	−	−	−	−	−	−	−
19	Pyridine	−	−	−	−	−	−	−	−
20	Phenol	−	−	−	−	−	−	−	−

[a] ++, the lowest detectable effective concentrations of tested chemicals were 10^3 to 10^5 times as much as that of the cognate ligand; +, 10^6 to 10^8 times; −, not detected.

NPnEOs）の代表として使用した。NP2EOは，親物質であるNPnEOsにも含まれているが，NPnEOsの水環境中での分解の最終産物の一つである。

エチレングリコール，チオ尿素，トルエン，2-アミノエタノール，DBS，1,3-ジクロロ-2-プロパノール，*p*-キシレンは和光純薬工業，N,N-ジメチルホルムアミド，ε-カプロラクタム，テレフタル酸（telephthalic acid；TPA），1,4-ジオキサン，アニリン，フェノールはキシダ化学，NP2EO，エチレンジアミン四酢酸，ジエチレントリアミン，メタクリル酸，アクリル酸，ヘキサメチレンジアミンは東京化成工業，ピリジンは林純薬工業から購入した。

標準リガンド及び被検化学物質は，DMSOに溶解後4 ℃で保存し，使用前にDMSOで段階希釈して用いた。

2.2 CoA-BAP法

本研究では，西川らが開発したCoA-BAP法[18]を用いて，供試化学物質のNRへの結合を評価した。CoA-BAP法は，生細胞を使用せずに，リガンドに依存したNRとコアクチベーターの相互作用をマイクロプレート上で検出する*in vitro*手法である。本手法では，大腸菌を用いて予め高発現させたNRリガンド結合領域（NR-LBD）とコアクチベーター（CoA）を使用する。NR-LBDを固定したプレートにCoAとリガンドを加えると，リガンドに依存してNRの立体構造が変化し，CoAがNR-LBDに結合する。CoAにはBAPが融合されているため，NRとCoAの相互作用の強さは，BAPのアルカリフォスファターゼ（AP）活性の強さとして測定できる。このAP活性は用量反応性を示し，検出感度が酵母two-hybrid法よりも高いことが確認されている[18]。また本法は，マイクロプレート上で操作するため，一度に多数の化学物質を試験することができる。さらに，NR-LBDとCoAをタンパクとして用いているため，これまで環境汚染化学物質のホルモン様活性の検出に汎用されてきた酵母法で問題視されてきた酵母細胞膜への透過性や酵母細胞に対する毒性による影響を回避することが可能である。

CoA-BAP法は，上述した原理に則って作製されたNuLigandシリーズ（マイクロシステムズ）を用いて行った。0.1 M炭酸緩衝液に溶解させた受容体を96穴マイクロプレートに分注し，4 ℃で一晩静置することでウェルに固定化させた。緩衝液A（Tris-HCl 20 mM, KCl 100 mM, EDTA 0.25 mM, glycerol 5%, dithiothreitol 0.5 mM, Tween 20 0.05%, pH 7.2）でウェルを3回洗浄後，緩衝液Aに懸濁させた30 $\mu g \cdot ml^{-1}$ のTIF2-BAP（PPARδ以外）或いはCBP-BAP（PPARδ）を100 μl分注し，適宜希釈した標準リガンド或いは被検物質を添加して，4 ℃で1時間静置した。緩衝液B（Tris-HCl 50 mM, KCl 100 mM, $MgCl_2$ 5 mM, Nonidet P-40 0.1%, pH 7.2）でウェルを3回洗浄後，発色基質（*p*-nitrophenylphosphoric acid 10 mM, Tris-HCl 100 mM, pH 8.0）を100 μl添加して，30 ℃或いは37 ℃で反応させ，405 nmの吸光度（A_{405}）を測定した。実験は全て3連で行い，平均と標準偏差（standard deviation；SD）を算出した。ある被検物質濃度におけるA_{405}値の平均- SDがDMSOのA_{405}値の平均+SDを上回り，それより高濃度でA_{405}値がさらに上昇した場合に陽性と判定した。

3．結　　果

CoA-BAP法により，10^{-9}-10^{-3} Mの範囲で，20種のPRTR化学物質の各種NRに対する結合性を調べた。17種類の化合物では，いずれのNRに対しても有意な結合性を示さなかったが，NP2EO，TPA，DBSの3物質はそれぞれ2，3，4種類のNRに結合した（**Table 1**）。NRで見ると，RXRα，PPARα，PPARδを除く5種類のNRに対して，少なくとも1種類の被検化学物質が結合した。活性を示した物質の用量反応曲線を受容体種ごとに**Fig. 1**に示す。

NP2EOは，ERαとPPARγに対して結合性を示した。ERαでは，標準リガンドであるE2に比べて10^3倍高濃度である10^{-6} Mから結合性が確認された（**Fig. 1A**）。一方，PPARγへの結合性が認められる濃度は標準リガンドに比べて10^6倍高濃度であり，活性は非常に低かった（**Fig. 1E**）。

TPAは，RARγ，VDR及びPPARγに対して結合性を示した。RARγに対する結合は10^{-7} Mから認められ，それより高濃度では濃度依存的に活性が上昇した（**Fig. 1C**）。VDRに対しては，10^{-8} Mで活性を示し，それより高濃度では，$1,25(OH)_2D3$と同様の割合で，濃度依存的に活性が上昇した（**Fig. 1D**）。PPARγにおいては，10^{-6} M以上の濃度で結合したが，それより高濃度における活性の増大は緩やかであった（**Fig. 1E**）。

DBSは，TRα，RARγ，VDR，PPARγの4種類のNRに結合した。TRαに対する結合性は，10^{-4} M以上の濃度でのみ認められた（**Fig. 1B**）。PPARγに対しては，10^{-6} Mで結合性を示した。その活性は10^{-6}-10^{-4} Mにおいて僅かずつ高まったが，10^{-4} M以上では変化しなかった。RARγとVDRに対しては，それぞれ10^{-6} M及び10^{-7} Mで結合性を示した。また，これらより高濃度では，それぞれの標準リガンドの用量反応曲線に類似した割合で濃度依存的に活性が増大した（**Fig. 1C, D**）。

4．考　　察

従来，内分泌攪乱活性に基づく環境汚染化学物質のリスク評価は，主にERとTRを対象として進められ，現在EDsとして疑いのある物質に挙げられているアルキルフェノール類やフタル酸エステル類，ビスフェノールA，農薬類などに対して数多くの知見が得られてきた。本研究で調べた20種類のPRTR化学物質の中で，NP2EOがERαに結合することが観察された。NP2EOは，酵母法[19]やマスのビテロゲニン遺伝子発現試験[20]においてエストロゲン様活性が確認されている。また，本研究で得られたNP2EOのERαに対する最少活性発現濃度は，既往研究[18]における4-ノニルフェノールのERαに対する最少活性発現濃度の1/100-1/10であり，これは酵母法における結果[19]と同等である。これらより，CoA-BAP法で得られたNRに対する結合性はある程度妥当なものであると考えられた。他方，TRαには，10^{-4} M以上の高濃度でDBSが結合する可能性のあることが示されたが，DBSを含むLASの甲状腺ホルモン様活性に関する報告はこれまでにない。

これまで，性ホルモン受容体とTR以外のNRは水環境中における化学物質のリスク評価に考慮されてこなかった。そこで本研究では，新たな試みとして，公共用水域に大量に排出されている化学物質を対象として，ER，TR以外のNRへの結合性を調べた。その結果，NP2EOがPPARγ，TPAがRARγ，VDR及びPPARγ，DBSがRARγ，VDR及びPPARγに結合する可能性のあることが確認された。特に，TPAとDBSのRARγ及びVDRに対する結合性は標準リガン

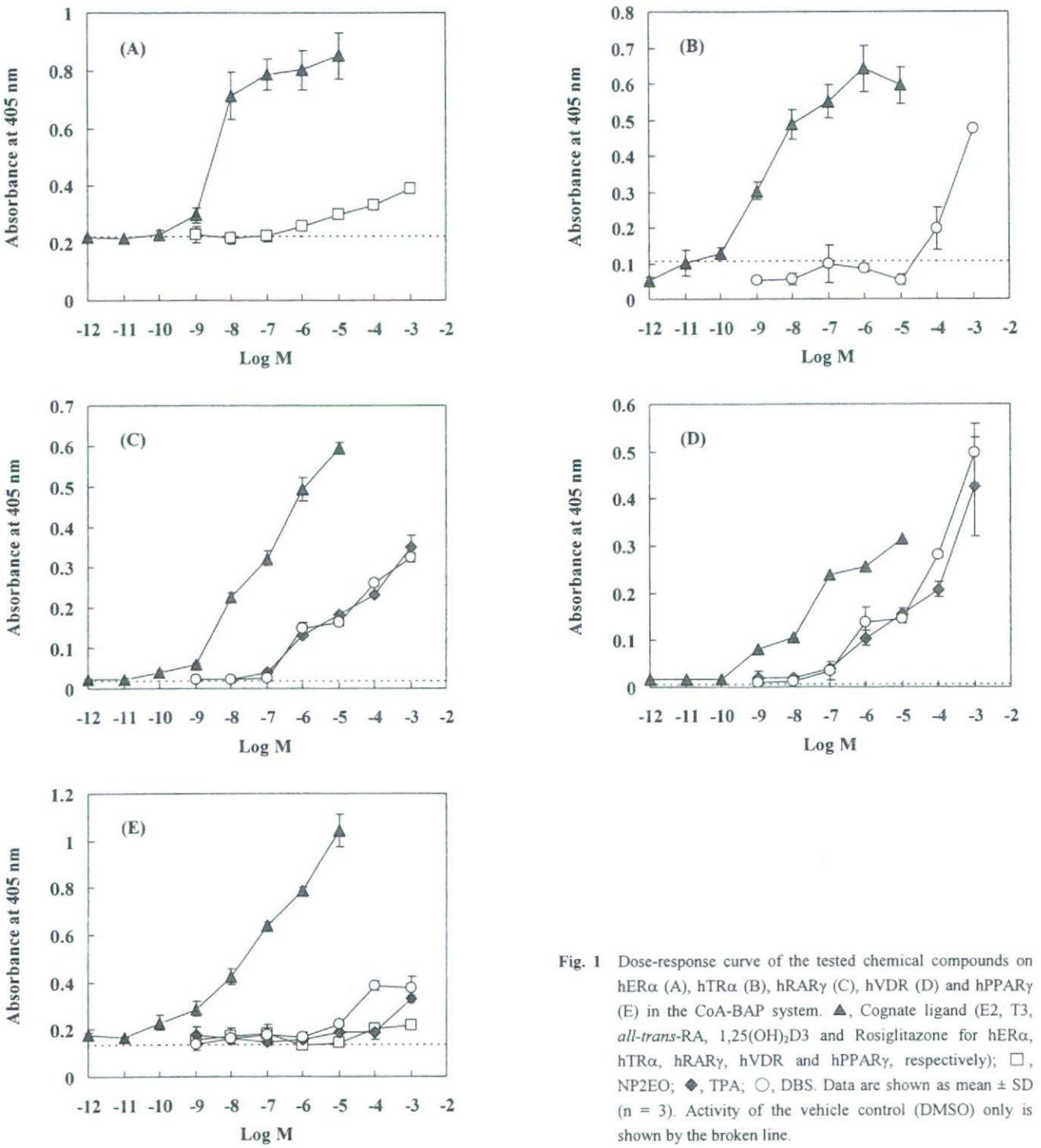

Fig. 1 Dose-response curve of the tested chemical compounds on hERα (A), hTRα (B), hRARγ (C), hVDR (D) and hPPARγ (E) in the CoA-BAP system. ▲, Cognate ligand (E2, T3, *all-trans*-RA, 1,25(OH)$_2$D3 and Rosiglitazone for hERα, hTRα, hRARγ, hVDR and hPPARγ, respectively); □, NP2EO; ◆, TPA; ○, DBS. Data are shown as mean ± SD (n = 3). Activity of the vehicle control (DMSO) only is shown by the broken line.

ドに類似した割合で濃度依存的に増大した(**Fig. 1C, D**)。これらの結果は，現在使用されている多種多様な化学物質の中に，48種類存在するNRのいずれか或いは複数に作用して，内分泌撹乱活性を示すものが数多く存在していることを強く示唆するものである。このため，今後のEDsの評価では，種々のNRを介した内分泌撹乱活性のスクリーニングを実施していくことが重要である。

本研究は被検化学物質のNRに対する結合性をスクリーニングしたものであり，陽性と判定されたケースで必ずしも生体影響が生じる訳ではない。しかし，本研究の結果はEDsによる内分泌撹乱作用の第一段階であるNRとの結合が生じる可能性を示すものであり，被検化学物質の潜在的な内分泌撹乱作用の可能性をある程度推測し得るものと言える。また，NRには種差があるため，ヒトNRを用いた本研究の結果が全野生生物種に当てはまる訳ではない。しかし，NRの種類によっては，異種間で高い保存性を示すものもあることから[9]，本研究の結果に基づき，ヒト以外の野生生物種を含め，被検物質の生物への悪影

響の可能性をある程度推察できるものと考えられる。そこで，以下では，本研究で得られた結果を基に，幾つかのNRに対する結合性が確認されたNP2EO，TPA及びDBSの潜在的な内分泌攪乱作用に関する推察を試みた。

RARは，脊椎動物の視覚や形態形成，発生，細胞分化，組織の恒常性に重要な役割を果たしている。RARのリガンドであるRAは，脊椎動物の催奇形物質であり，その過剰摂取は多種多様な奇形を発生させる[21,22]。1990年代から北米で観察されているカエルの奇形にも，水環境汚染化学物質によるRARシグナル伝達系の攪乱が関係していることが指摘されている[23,24]。VDRは，カルシウムの恒常性，骨代謝，及び他の重要な生物作用（細胞分化の誘導，細胞増殖阻害，免疫修飾，他のホルモン系の制御など）において中心的な役割を担っている[25]。PPARγは，主に脂肪とグルコースの代謝に重要な役割を果たしており，種々の臓器において抗発癌作用を発現する[26]。また，そのアゴニストは，臨床でII型糖尿病の治療薬として使用されている。現在のところ，環境中でVDR或いはPPARγを介したものと考えられる悪影響の観察事例はない。

RARγ[27]とPPARγ[26]は，RXRとヘテロ二量体を形成し，各々の標的遺伝子を転写活性化する。しかし，これらの受容体への結合性が認められたNP2EO（PPARγ），TPA（RARγ，PPARγ），DBS（RARγ，PPARγ）は，いずれもRXRαには結合しない（**Table 1**）。NRが二量体を形成して活性化されるシグナル経路においては，リガンドが二量体を成すNRの片方にしか結合しない場合の影響は，双方に結合する場合に比べて小さい可能性が指摘されている[9]。このことから，NP2EO，TPA，DBSが単独でRARやPPARγを介するシグナル伝達系を攪乱する可能性は小さいことが示唆された。しかし，有機スズ化合物のようにRXRαに結合する化学物質が共存する環境下では，RARやPPARγのシグナル伝達系への悪影響が生じる可能性もあり得る。ただし，RARとPPARγに対する最少活性発現濃度が環境水中濃度に比べて高いことから（NP2EOで10^4倍以上[28]，TPAで100倍以上[29]，DBSで5倍以上[30]），現状ではこれらの物質が生物に悪影響を及ぼしている可能性は低いものと考えられる。

他方，VDRは，ホモ二量体，或いはRARかRXRとのヘテロ二量体を形成し，VD応答遺伝子を転写制御する[31,32]。VDRへの結合が確認されたTPAとDBSはRXRに結合しないことから，上述したRAR及びPPARγのケースと同様に，VDR-RXRヘテロダイマーを介するVDRシグナル伝達機能への悪影響の可能性は低いものと予想される。一方，TPAとDBSは，RARγとVDRの両受容体に対する結合性が濃度依存的に増大したことから，それぞれ単独でVDRホモ二量体或いはVDR-RARヘテロ二量体に強く作用する可能性がある。TPAは，混餌投与によって，Fischer-344ラット離乳児に対して，TPAカルシウムを主成分とする膀胱結石を形成し，膀胱移行上皮の肥厚化と高カルシウム尿症を引き起こすことが報告されている[33]。また，DBSを含むLASは，経口投与によって哺乳動物胎児に骨化遅延を引き起こすことが明らかにされている[34]。すなわち，両物質が有する生体毒性は，VDRシグナル伝達系が関わる生体内機能と興味深い一致を示している。以上のことから，TPAとDBSは，急性毒性を示さない濃度において，VDRホモ二量体或いはVDR-RARヘテロ二量体に作用することでVDRシグナル伝達機能を乱し，生体に悪影響を及ぼす可能性のあることが示唆された。しかし，TPAは，環境水中濃度がRARγ及びVDRへの最少活性発現濃度よりもそれぞれ100倍及び10倍以上低く，また，生分解性が良好で残留性が低いことから[29]，実環境中で活性が発現する可能性は高くないものと推察される。他方，DBSは，環境水中濃度がRARγ及びVDRに対する最少活性発現濃度と同程度の場合もあることから[30]，活性発現の可能性を完全には否定できないが，水環境中での生分解性が高く，数時間～数日間で消失することから[30]，リスクはさほど高くないものと推定される。以上のように，現状のデータからでは，TPAやDBSが単独で野生動物のVDRシグナル伝達機能に悪影響を及ぼす可能性は低いと考えられるものの，これらが共存した場合には，相乗的な作用が生じることも否定できない。また，環境中への排出実態が明らかでない微量化学物質の中にもNRに結合性を示すものが存在すると推測されることから，多様なNRへの結合を介したリスクについて評価していくことが望まれる。

5．ま と め

本研究では，公共用水域への排出量の多いPRTR第1種指定化学物質20種のNR結合性を調査した。その結果，これまでに内分泌攪乱活性が疑われてこなかったTPAとDBSが複数のNRに結合する可能性のあることが示された。このことは，現在の評価体系で断定されている，或いは疑われているEDs以外の物質が，性ホルモン受容体やTR以外のNRを通じて，内分泌攪乱作用を示す可能性のあることを強く示唆している。今後の内分泌攪乱作用に関するリスク評価では，環境中に排出される可能性のある多様な人工化学物質を対象とし，種々のNRへの結合を介したリスクについて，網羅的，総合的に評価していくことが重要である。

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AIB1 Promotes DNA Replication by JNK Repression and AKT Activation during Cellular Stress

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Amplified in breast cancer 1 (AIB1) is a member of the p160 family of nuclear receptor coactivator protein. Recent studies have reported that high-level AIB1 production is involved in the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway for progression to malignant carcinoma in a steroid-independent manner. Here we demonstrate that, in AIB1-knockout DT40 chicken B-lymphocytes, loss of AIB1 results in induction of phosphorylation of c-Jun N-terminal kinase (JNK) and c-Jun, in addition to the inhibition of DNA replication. In contrast, high-level AIB1 production prevents proapoptotic activation of the JNK/c-Jun signal transduction pathway and induces DNA replication through phosphorylation of the Akt/p65 NF-κB subunit RelA under cellular stresses such as UV irradiation or serum deprivation. Moreover, we have found that AIB1 is essential for the phosphorylation of histone H3 at serine 10, which is associated with the signal transduction to chromatin, leading to the transient expression of immediate-early genes in response to UV stimulation. Our results therefore suggest that AIB1 directly links to cell cycle control mechanisms in concern with the balance between apoptosis and proliferation.

Key words: amplified in breast cancer 1, cellular stress, DNA replication, phosphorylation, signal transduction.

Abbreviations: AIB1, amplified in breast cancer 1; Akt, cellular homolog of v-akt oncogene; CARM1, coactivator-associated arginine methyltransferase 1; CBP, cyclic AMP response element binding protein; CDK, cyclin dependent kinase; ER, estrogen receptor; ERK, extracellular signal–regulated kinase; FCS, fetal cauf serum; FACS, fluorence-activated cell sorting; GSK3, glycogen synthase kinase 3; HAT, histone acetyltransferase; HER-2, human epidemal growth factor receptor-2; JNK, c-Jun amino-terminal kinase; MAPK, mitosis-activated protein kinase; NF-κB, nuclear factor-κB; PAS, Per/Arnt/Sim; PI3K, phosphatidylinositol 3 kinase; RSK2, ribosomal S6 kinase 2; SRC-1, steroid receptor coactivator-1; TIF2, transcriptional intermediary factor 2; TUNEL, terminal deoxynucleotidyl transferase–mediated nick end label.

The nuclear receptor coactivator known as AIB1 (also called p/CIP, ACTR, RAC3 and SRC-3) is a member of the p160 nuclear receptor coactivator family. This family contains SRC-1 (steroid receptor coactivator-1) and TIF2 (transcriptional intermediate factor-2) that interact with the general transcriptional coactivators CBP, p300 and p/CAF (*1–8*). These coactivator complexes possess intrinsic histone acetyl transferase activity and are responsible for the remodelling of chromatin and modification of components of the transcription machinery (*9, 10*).

AIB1 increases estrogen-dependent transcriptional activation by interaction with estrogen receptor (ER) α in a ligand-dependent manner. Furthermore, AIB1 mRNA and protein have been shown to be amplified and overexpressed in primary human breast and ovarian cancer cell lines, in which transcription is upregulated and the AIB1 gene on chromosome 20q12 is amplified (*1*). Recent studies report that high levels of AIB1 production are relates to both a high DNA-synthesis phase fraction and HER-2/*neu* production with p53 mutations in breast cancer, which is a disease characterized by an imbalance between cell division and cell death (*11, 12*). Her-2/*neu* protein activates the PI3K (phosphoinositide 3-kinase)/Akt (also known as protein kinase B, PKB) pathway, which, through NF-κB activation, plays an important role in preventing cells from undergoing apoptosis (*13, 14*). More recently, it has been shown that overexpression of AIB1 in the mammary gland leads to activation of the PI3K/Akt pathway, with IGF-1 signaling (*15*). Because AIB1 (RAC-3) has also been shown to interact with NF-κB and enhance its transcriptional activity (*16, 17*), it has been suggested that AIB1 is an altered regulator for the mechanism by which constitutive activity of an NF-κB–dependent promoter is involved in chemotherapeutic resistance in ER-negative cancer cells (*18, 19*). However, the biological function of AIB1 in the signal transduction pathways influenced by complex cascades of phosphorylation events triggered by exposure to cellular stress is not completely understood. Therefore, we focused our attention on AIB1 activity, to determine whether this protein regulates the antiapoptotic process or perturbs signal integration in response to cellular stress.

Importantly, c-Jun N-terminal kinase (JNK) has also been shown to be a key regulator of programmed cell death and part of a subfamily of the mitogen-activated protein kinase (MAPK) superfamily (*20*). Recent studies indicate that JNK activation contributes to

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TNF-α–induced apoptosis in the absence of NF-κB activation, and that NF-κB–mediated inhibition on JNK activation is important for cell survival (*21–24*). Here we show that, in wild-type DT40 cells, high-level production of AIB1 suppresses the phosphorylation of JNK and c-Jun, the main physiological substrate of the JNK kinase, in response to cellular stress such as serum deprivation or UV irradiation. In contrast, loss of AIB1 leads to inhibition of activation of the Akt/p65 signaling pathway and suppresses DNA synthesis.

Finally, we found that AIB1 enhances the induction of phosphorylation of histone H3 at serine 10 but not the acetylation of histone H3 at lysine 9 or lysine 14 in response to UV stress. Phosphorylation of histones provides motifs for the recruitments of chromatin modifying or remodelling complexes, including coactivators which are linked to cellular processes such as transcription, DNA replication, DNA repair and apoptosis in the stress response (*25*, *26*). Because AIB1 enhances the induction of phosphorylation of histone H3 at serine 10, it plays a critical role as a transcriptional modifier that is recruited for chromatin remodelling in response to cellular stresses. These results indicate that changes in AIB1 production may determine cell fate in association with the balances between the Akt/p65 and JNK/c-Jun signaling pathways in cellular stress responses.

EXPERIMENTAL PROCEDURES

Cell Culture—DT40 cell lines were maintained in RPMI 1640 (Nikkenseibutsu) medium supplemented with 10% fetal calf serum (FCS; Gibco BRL) and 1% chicken serum (JRH Bioscience) at 39.5°C in a humidified atmosphere with 5% CO_2. Cell density was maintained at 0.1–1.0/10^6 ml by splitting the culture daily.

Construction of Targeting and Expression Vectors—A chicken AIB1 (*GdAIB1*) partial cDNA fragment from the bHLH/PAS domain was amplified from chicken brain cDNA by RT-PCR with primers (5′-aaggaaaaactatttccagtgagatgatgttc-3′, 5′-cgaattgtatcctaaagccaggtctcagg-3′). We then used 5′ and 3′ RACE on chicken brain cDNA to isolate the entire open reading frame of *GdAIB1*. To construct the *GdAIB1* expression vector, chicken *AIB1* cDNA was inserted into an expression vector containing the chicken β-actin promoter. We then isolated 6.5 kb of the partial chicken genomic *GdAIB1* locus from DT40 genomic DNA by long-range PCR. Chicken *AIB1* targeting constructs were made by replacing the genomic sequence containing the sequence encoding amino acids 122 to 156 with hygromycin or histidinol selection marker cassettes.

Generation of AIB1 −/− Clones—10^7 DT40 cells were suspended in 0.5 ml PBS containing 30 μg of linearized plasmid for the transfection and electroporated with a Gene Pulser apparatus (BioRad) at 550 V and 25 μF. Following electroporation, cells were transferred into 20 ml fresh medium and incubated for 24 h. Cells were then resuspended in 80 ml medium containing hygromycin (2.5 mg/ml, Calbiochem) or L-histidinol (1 mg/ml, Calbiochem) and divided into four 96-well plates. After 7 to 10 days, drug-resistant colonies were selected. Disruption of the gene was confirmed by Southern blot analysis of genomic DNA.

Cell Cycle Analysis—A total of of 2×10^5 cells were treated with 5-bromodeoxyuridine (BrdU; 10 μM, Sigma) for 10 min and the subconfluent cells harvested. After fixation with 70% ethanol, the cells were incubated overnight at −20°C. The next day, cells were collected and resuspended in 2 N HCl with 0.5% Triton X-100 for 30 min at room temperature; this was followed by neutralization with 0.1 M $Na_2B_4O_7$. Cells were then collected and incubated with anti-BrdU antibody (Becton-Dickinson) for 30 min in the dark at room temperature. The cells were washed with PBS and stained with FITC-labeled goat anti-mouse Abs (Jackson) for 30 min at room temperature in the dark. The cells were resuspended in PBS containing propidium iodide (5 μg/ml, Sigma). The filtered cells were analyzed by fluorescence-activated cell sorter (FACScan, Becton-Dickinson). The distribution of cells in each phase of the cell cycle was determined by using Cell Quest software (Becton-Dickinson).

TUNEL Assay—DT40 cells were harvested at the designated time points and fixed in 70% ethanol in PBS. The fixed cells were then incubated for 30 min at 4°C and permeabilized with 0.2% Triton X-100 in PBS for 5 min. For apoptosis analysis, the cells were examined by the TUNEL technique, as described in the instructions supplied with the apoptosis detection system (Takara). Localized green fluorescent apoptotic cells were detected by fluorescence microscopy. The percentage of FITC-positive cells in the apoptotic fraction was determined in a fluorescence-activated cell sorter (Becton Dickinson).

Western Blot Analysis—Cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl at pH 7.5, and 1% NP-40) supplemented with protease inhibitors, aprotinin (1 μg/ml), and leupeptin (2 μg/ml). Lysates were centrifuged to clear cell debris, and then 30 μg of the total protein were size-fractionated by SDS-PAGE gel (7.5% to 15%). After electrophoresis, proteins were transferred to PVDF membranes (BioRad), blocked in PBS containing 0.2% Tween 20 and 3% bovine serum albumin, and probed with first antibody in PBS containing 0.2% Tween 20 and 1% bovine serum albumin. Detection of the immune signal was performed with the chemiluminescence detection system (Amersham Biosciences) and then quantified using densitometry (Molecular Dynamics).

Antibodies—The amino-terminal portion of chicken AIB1 (amino acids 1 to 250) was expressed in *E. coli* as a histidine tagged fusion protein and purified by affinity chromatography using Ni-agarose (Qiagen). The purified protein was then injected into rabbits to prepare specific antiserum. Rabbit antibodies of anti-phospho-Akt, anti-Akt, anti-phospho-p65, anti-p65, anti-phospho-JNK, anti-JNK, anti-phospho-c-Jun, anti-c-Jun, anti-phospho-p38, and anti-phospho-p44/42 ERK1/2 were purchased from Cell Signaling Technology. Mouse antibodies of anti-p38 and anti-ERK1 were purchased from BD Bioscience. Rabbit anti-histone H3 phosphorylated at Ser10 or Ser28 and rabbit anti-histone H3 acetylated at Lys9 or Lys14 antibodies were purchased from Upstate Biotechnology.

Immunoprecipitations and In Vitro Akt Kinase Assay—Cells were disrupted with cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% DOC, and 0.1% SDS) containing protease inhibitors, aprotinin (1 μg/ml) and leupeptin (2 μg/ml). Approximately 200 μg of protein lysate was incubated with anti-chicken AIB1

antibody and anti-Akt antibody overnight at 4°C with end-over-end rotation, followed by an additional 2 h of incubation with protein A sepharose beads (Amersham Biosciences). The beads were then washed three times with cold RIPA buffer before being boiled in SDS-PAGE sample buffer. *In vitro* Akt kinase assay was performed for 30 min at 30°C in 40 μl of reaction volume containing 30 μl of immunoprecipitates in kinase buffer with 200 μM ATP. GSK-3 fusion protein (Cell Signaling Technology) was used as a substrate for Akt kinase activity. The reactions were terminated with 20 μl of SDS sample buffer and subjected to Western blotting using anti-phospho-GSK antibody (Cell Signaling Technology).

Northern Blot Analysis—Total RNA was isolated with Trizol reagent (Invitrogen). For generation of each probe, 1 μg of total RNA was used in reverse transcription reactions, as described by the manufacturer. The resulting total cDNA was then used in the PCR to estimate mRNA levels. The mRNA level of GAPDH was used as internal control. PCR was carried out with Taq polymerase, and the conditions were as follows: pre-denaturing at 94°C for 3 min, then 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The oligonucleotide primers used to generate these probes were as follows: AIB1 (GenBank accession number: XM417385), 5′-attacctgcattcagagaagaat-3′ and 5′-tcttctctcattgtctacacaa-3′; chicken cyclin D1 (U40844), 5′-tttacaccgacaactccatc-3′ and 5′-gtgataggaaatgtgtgagg-3′; chicken cyclin D2 (U28980), 5′-ccatcaatgatagcaactgg-3′ and 5′-aaaataaaaggggtgggag-3′; chicken cyclin E (U28981), 5′-cttcaccgctaccaattctg-3′ and 5′-caaactggtg-caactttggt-3′; chicken E2F1 (X89245), 5′-ggatccccggca-gaggggca-3′ and 5′-ctccaggacattggtgatgt-3′; GAPDH (NM204305), 5′-accactgtccatgccatcac-3′ and 5′-tccacaa-cacggttgctgta-3′. Each total RNA (30 μg) was run on a 1.0% formaldehyde gel and transferred to a Hybond N+ nylon membrane (Amersham) using a Turbo blotter system (Schleicher & Schuell). DNA probes were labeled with [α-^{32}P] dCTP (Amersham Biosciences) using a random labeling kit (Takara). The membrane was hybridized with labeled DNA probes by QuikHyb hybridization (Stratagene) at 65°C for 2 h and then developed for autoradiography.

Histone Extraction—Cells were centrifuged, the medium discarded and the cells washed twice with PBS (pH 7.4). The cells were suspended in 5 to 10 volumes of lysis buffer (10 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 1.5 mM PMSF). The samples were incubated on ice for 30 min and centrifuged at 1,000 × *g* for 10 min at 4°C. The supernatant was discarded, and the histones in the pellets were extracted by 0.2 M HCl solution. The samples were centrifuged at 12,000 × *g* for 10 min at 4°C after incubation on ice for 30 min. The histones were then precipitated from acid solution with 5 volumes of cold acetone.

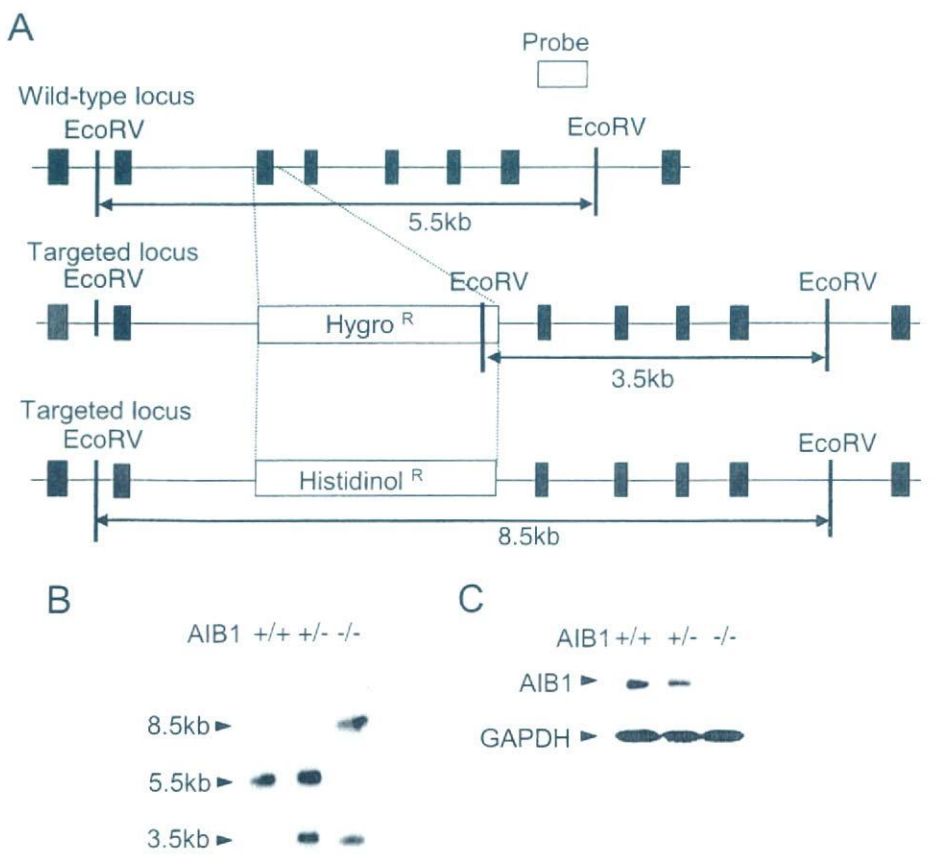
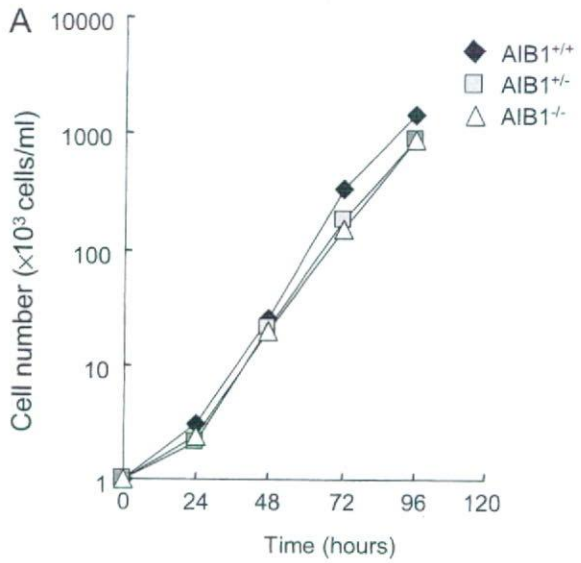
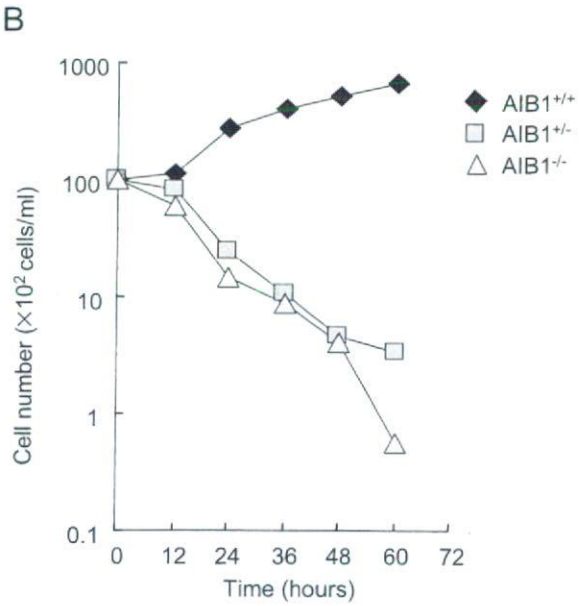
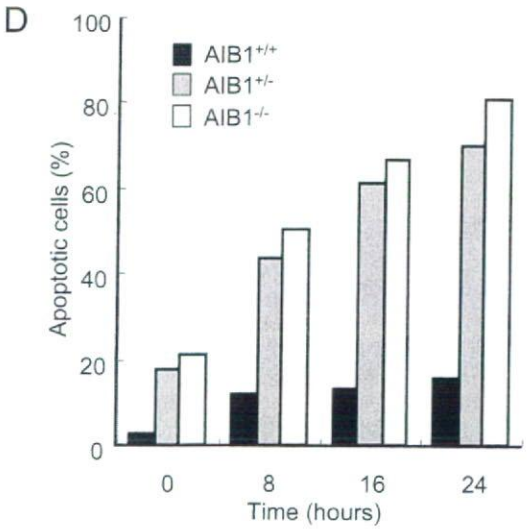
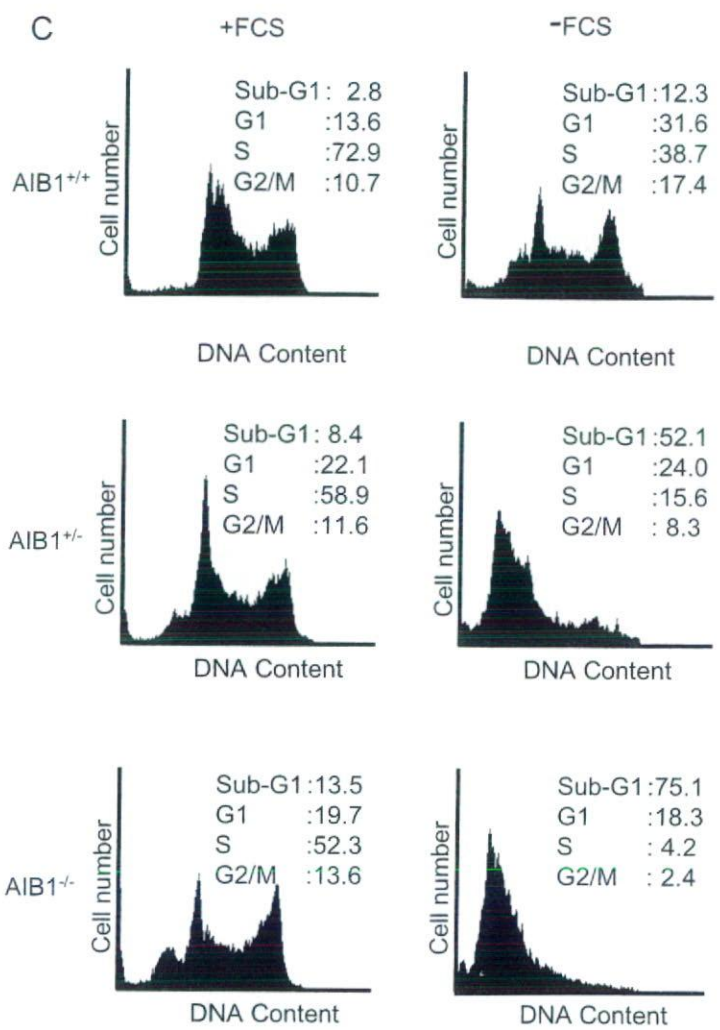


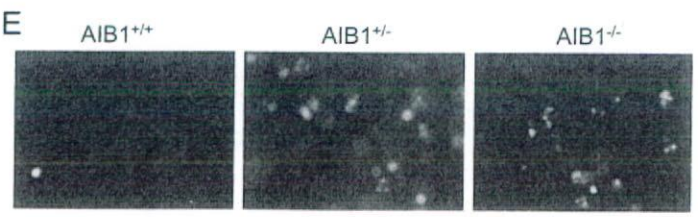
Fig. 1. **Generation of AIB1-knockout DT40 cells.** (A) Schematic representation of the targeting vectors. The configuration of the wild-type allele is shown at the top. In the targeting vectors, the exon encoding the Per-Arnt-Sim (PAS) domain is replaced by a hygromycin or histidinol resistance gene cassette. Solid boxes indicate positions of exons deduced from the cDNA sequence. The location of the external probe used to confirm correct targeted events and the location of the relevant *EcoRV* recognition sites are indicated. (B) Southern blot analysis of targeted integration. A DT40 cell in which one *AIB1* allele had been disrupted by the targeting construct of AIB1-hygromycin, was transfected with the second construct of AIB1-Histidinol. Genomic DNAs from untransfected DT40 cells (+/+) and doubly resistant clones (–/–) were digested with *EcoRV* and hybridized with the probe shown in (A). (C) Western blot analysis of wild-type, AIB1$^{+/-}$ and AIB1$^{-/-}$ DT40 cells. GAPDH protein was detected as an internal control.

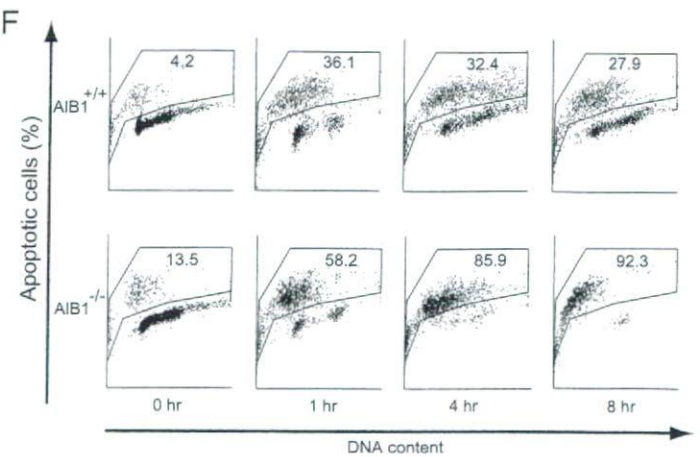

A
Cell number (×10³ cells/ml)
10000
1000
100
10
1
0
24
48
72
96
120
Time (hours)
AIB1+/+
AIB1+/-
AIB1-/-


B
Cell number (×10² cells/ml)
1000
100
10
1
0.1
0
12
24
36
48
60
72
Time (hours)
AIB1+/+
AIB1+/-
AIB1-/-


D
Apoptotic cells (%)
100
80
60
40
20
0
0
8
16
24
Time (hours)
AIB1+/+
AIB1+/-
AIB1-/-


C
+FCS
-FCS
AIB1+/+
AIB1+/-
AIB1-/-
Cell number
DNA Content
Sub-G1: 2.8
G1 :13.6
S :72.9
G2/M :10.7
Sub-G1:12.3
G1 :31.6
S :38.7
G2/M :17.4
Sub-G1: 8.4
G1 :22.1
S :58.9
G2/M :11.6
Sub-G1:52.1
G1 :24.0
S :15.6
G2/M : 8.3
Sub-G1:13.5
G1 :19.7
S :52.3
G2/M :13.6
Sub-G1:75.1
G1 :18.3
S : 4.2
G2/M : 2.4


E
AIB1+/+
AIB1+/-
AIB1-/-


F
Apoptotic cells (%)
AIB1+/+
AIB1-/-
4.2
36.1
32.4
27.9
13.5
58.2
85.9
92.3
0 hr
1 hr
4 hr
8 hr
DNA content

RESULTS

Generation of AIB1-Knockout DT40 Cells—To investigate the biological function of AIB1 in the signal transduction pathways triggered by cellular stress, we generated a model for the elimination of AIB1 production by using DT40 B-lymphocytes that constitutively express high levels of *c-myc* as a result of transformation by an avian leukosis virus (*27*). Because of their high rate of homologous recombination in vertebrate cells, DT40 cell lines have also been used as models for establishing strategies to identify genes that encode undiscovered components of a process or a pathway (*28*). We previously reported the cloning of a chicken homologue of AIB1 that exhibits 74.4% amino acid sequence similarity to human AIB1 (*29*). A full-length transcript of the gene encoding chicken AIB1 was isolated and found to encode a 1,399–amino acid protein. An AIB1 deletion construct was generated (Fig. 1A) and a portion of the AIB1 genomic locus was replaced with a hygromycin resistance gene and a histidinol resistance gene. The targeted homologous recombination in DT40 cells was confirmed by Southern blot analysis of genomic DNA (Fig. 1B). The deleted region encoded amino acid residues 122 to 156, including the PAS domain. AIB1-knockout DT40 cells were detected by Western blot analysis using anti-AIB1 antibody (Fig. 1C).

AIB1 Promotes Cell Survival in Response to Cellular Stress in DT40 Cells—To determine the roles of AIB1 in mechanisms mediated by exposure to cellular stress in cancer cells, we examined whether loss of AIB1 was associated with changes in the regulatory mechanism of cell proliferation and cell death. Under standard culture conditions, we detected no significant difference in the rates of cell proliferation of wild-type and AIB1-knockout DT40 cells (Fig. 2A). However, AIB1-knockout cells were more susceptible to cell death than were wild-type cells in response to cellular stresses such as serum starvation. Usually, DT40 cells were maintained in medium with 10% FCS and 1% chicken serum. After deprivation of FCS, survival number of AIB1-knockout cells rapidly decreased in contrast with wild type cell (Fig. 2B). The heterozygote also manifested considerable decrease in cell number, suggesting that gene dosage of AIB1 is crucial for cell survival under serum starvation (Fig. 2B). FACS analysis revealed that 75.1% of the AIB1-knockout cells were in the sub-G1 fraction (reflecting cell death), as compared to 12.3% for the wild-type cells under serum starvation (Fig. 2C). In contrast, the S-fraction (indicating DNA synthesis) was greater in wild-type cells than in AIB1-knockout cells (Fig. 2C). In order to confirm that these cell death were apoptosis, we carried out TUNEL analysis. When cells were stained by the TUNEL after deprivation of FCS, the double-stained cells (reflecting apoptosis) increased much more in $AIB1^{+/-}$ and $AIB1^{-/-}$ DT40 cells than in wild-type DT40 cells (Fig. 2, D, E). We observed similar results in the responses to not only serum starvation but also other forms of cellular stress, such as UV irradiation (10 J/m^2) (Fig. 2F) and culture at low temperature (data not shown). These results revealed that loss of AIB1 increased apoptosis in AIB1-knockout cells under cellular stress conditions.

Deletion of AIB1 Enhances Stress-Induced JNK/c-Jun Activation and Prevents Akt/p65 Activation—Signal cascades in response to cell survival have been linked to cancer and inflammatory disease. Previous studies have shown that cellular responses are regulated by the signaling pathways that lie downstream of the Ras induced by cross talk between the Raf-MER-ERK and PI3K-Akt pathways in breast cancer cells (*21*, *22*). Moreover, the PI3K/Akt/NF-κB pathway plays an important role in preventing cells from undergoing apoptosis and contributes to the pathogenesis of malignancy (*23*). Recently, cell proliferation and survival were reported to require activation of the PI3K/Akt pathway, which has been implicated in the control of Myc protein stability (*24*). The most recent study in transgenic mice also implicated overexpression of the *AIB1* gene in the etiology of breast cancer (*15*). However, these findings and our results in Fig. 2 raise the possibility that increased AIB1 production serves to promote cell survival through the PI3K/Akt pathway in response to cellular stress. Cell survival in response to stressful stimuli has been implicated in the activation of many signal transduction pathways, such as the p38 mitogen-activated protein kinase (MAPK) pathway, the stress-activated JNK pathway, and the PI3K/Akt pathway (*30–32*). Therefore, we investigated how signal pathways might be involved in AIB1-mediated cell survival in response to cellular stress. For Western blot analysis, wild-type and AIB1-knockout DT40 cells were cultured under normal conditions or cellular stress conditions. At first, stressful stimuli such as serum starvation or UV irradiation induced phosphorylation of NF-κB subunit p65 and Akt in wild-type DT40 cells (Fig. 3A). In contrast, phosphorylation of p65 and Akt were abolished in AIB1-knockout cells (Fig. 3A). To confirm the Akt activation under stress conditions, Akt activity was detected by an

Fig. 2. **AIB1 promotes cell survival in response to serum starvation and UV irradiation.** (A) Growth curves of wild-type, $AIB1^{+/-}$ and $AIB1^{-/-}$ DT40 cells under standard conditions. Cells were cultured in RPM1640 medium supplemented with 10% fetal calf serum (FCS) and 1% chicken serum at 39.5°C. Representative growth curves correspond to the indicated cell cultures. The number of cells was counted every 24 h by FACS with polybeads as the internal standard. Each experiment was conducted three times, and each time point was determined in triplicate. (B) AIB1 was required for cell survival under serum-starved conditions. Cells were cultured at 39.5°C in fresh RPM1640 medium supplemented with 1% chicken serum, after being maintained continuously with 10% FCS and 1% chicken serum. The number of cells was counted every 12 h. (C) Cell cycle analyses of wild-type, $AIB1^{+/-}$, and $AIB1^{-/-}$ DT40 cells in the presence or absence of FCS over 24 h. The DNA content of the cultured cells was examined by propidium iodide staining. The populations of the sub-G1, G1, S, and G2/M fractions are indicated by percentages. (D) Wild-type DT40 cells were inhibited from undergoing apoptosis under serum starvation, as measured by TUNEL analysis. Cells were cultured in the absence of FCS for the periods indicated. The cells were then stained for TUNEL analysis. Double-stained cells were counted as apoptotic and are shown as the percentages in three independent experiments. (E) DNA breaks, characteristic of apoptosis, were detected under fluorescent microscope by TUNEL analysis. Cells were harvested after being cultured for 8 h in the absence of FCS. (F) DNA replication was induced in wild-type DT40 cells under the stimulation of UV-irradiation, as measured by TUNEL analysis. After UV-irradiation (10 J/m^2) the cells were cultured for the periods indicated and then stained for TUNEL analysis. Double-stained cells were counted as apoptotic and are shown as percentages from three independent experiments.

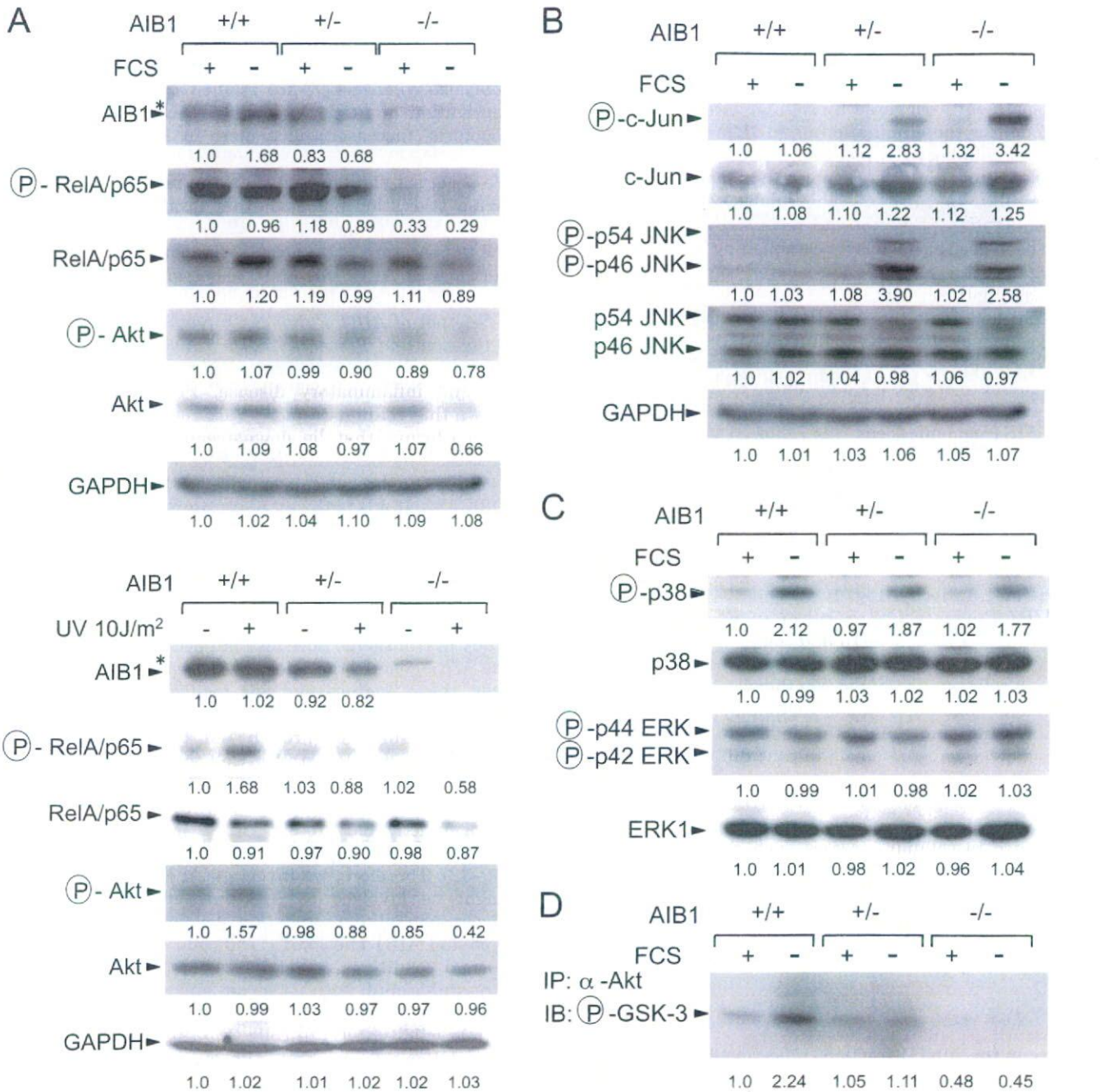


Fig. 3. **Activation of AIB1 triggers Akt survival pathway and blocks JNK-mediated cell death.** (A) Serum starvation and UV irradiation of wild-type DT40 cells induced Akt and p65 phosphorylation. The levels of production of proteins and phosphoproteins in the Akt/NF-κB cell-survival pathway were indicated by immunoblot analysis using 30 μg cell extracts prepared from wild-type, $AIB1^{+/-}$, or $AIB1^{-/-}$ DT40 cells cultured for 8 h in the presetnce or absence of FCS, with or without stimulation of UV-irradiation (10 J/m^2). The "P" in the circles indicates phosphorylation. The asterisk indicates that AIB1 antibody cross-reacted with the unspecific cytoplasmic protein. (B) Phosphorylation of JNK and c-Jun were induced by serum starvation in AIB1-knockout cells. The levels of production of protein and phosphoprotein in the JNK-mediated apoptosis pathway are indicated as shown in (A). (C) The presence of AIB1 protein did not affect the activation of the ERK or p38 MAPK pathways. (D) Serum starvation of wild-type DT40 cells induced Akt kinase activity. *In vitro* kinase assay was performed with immunoprecipitated Akt. Akt kinase activity is indicated by immunoblot analysis with glycogen synthase kinase-3 as a substrate. Western blots were quantified by densitometry and relative intensities of each band are shown.

in vitro assay of Akt-catalyzed phosphorylation of GSK3 (Fig. 3D). The catalytic activity of Akt was increased in wild-type DT40 cells by serum starvation. The activation of the PI3K/Akt pathway might be related to the cell survival of wild-type DT40 cells under stress conditions. Second, serum starvation induced phosphorylation of c-Jun and JNK in AIB1-deficient cells, not in wild type cells (Fig. 3B). Because both of $AIB1^{+/-}$ and $AIB1^{-/-}$ cells were killed by FCS deprivation (Fig. 2B), there are good correlation between the activation of JNK/c-Jun pathway

and the cell death. The stress-activated JNK pathway might be related to the cell death of AIB1-deficient cells under stress condition. On the other hand, there was no difference in the p38 MAPK/ERK pathway (Fig. 3C). Taken together, our data suggest that AIB1 acts as a molecular link between Akt/p65-induced cell survival and JNK/c-Jun-regulated cell death in response to cellular stress.

In Fig. 3A, a band was observed in the panel for AIB1$^{-/-}$ cells. This weak signal was found in all lanes using anti-AIB1 antibody, although it was difficult to identify because it was just above the specific band. Possibly, our AIB1-antibody might cross-react to the closely related protein such as TIF2 or SRC1.

Cellular Stress Induces Upregulation of AIB1 Gene Expression—To test directly whether cellular stress causes increased AIB1 gene expression by modulating Akt function or cell cycle regulators in G1/S transition, we examined the levels of expression of the mRNAs of various cell cycle regulators by Northern blotting in wild-type or AIB1-knockout DT40 cells after stimulation by serum starvation (Fig. 4). The mRNA levels of all the regulators investigated in AIB1-knockout cells were decreased efficiently by serum starvation. In contrast, the mRNA levels of AIB1, Akt, and RSK2 in wild-type DT40 cells were increased significantly by serum deprivation. No notable increases in mRNA levels of the G1/S-cell cycle regulators cyclin D1, cyclin D2, cyclin E and E2F1 were found in wild-type DT40 cells under serum starvation. These data indicate that, under cellular stresses such as serum starvation, high levels of AIB1 mRNA are mediated by Akt or RSK2, but not by activation of the regulators of cell cycle progression in the G1/S phase. Accordingly, cellular stress-induced AIB1 amplification may enhance cell survival and DNA replication through coordinated upregulation of the Akt signaling pathway.

AIB1 Is Essential for Induction of Akt-Dependent DNA Replication in Response to Cellular Stress—To assess the role of AIB1 in mediating Akt-activated DNA replication in response to cellular stress, we analyzed the contribution of phosphorylated Akt at designated time points after synchronization of the cell cycle by treatment with hydroxyurea, which arrests the cycle in the early S-phase. While we observed no differences in the low levels of Akt phosphorylation during synchronization, phosphorylation of Akt was blocked in AIB1-knockout DT40 cells, unlike in wild-type cells, after release of the cell cycle arrest. In contrast, induction of Akt phosphorylation was significantly increased in wild-type cells when they were released from arrest and progressed further into S phase (Fig. 5A). By treatment with the pharmacological agent LY294002, an inhibitor of PI3K/Akt kinase, we next investigated whether the requirement for AIB1 in DNA replication was dependent on Akt in stressed cells. Wild-type DT40 cells with LY294002 had marked decreased numbers of BrdU-positive cells (Fig. 5B), suggesting that, in wild-type cells, inhibition of cell cycle progression into S phase occurred by the inhibition of Akt, in the same way as in AIB1-knockout cells. Inhibition of MAPK activity by treatment with PD98059 did not lead to a marked decrease in DNA replication in response to serum starvation in wild-type DT40 cells (data not shown). Thus, these results indicate that induction of Akt phosphorylation as a consequence of progression into S phase caused a requirement for AIB1 in DNA replication in response to cellular stress.

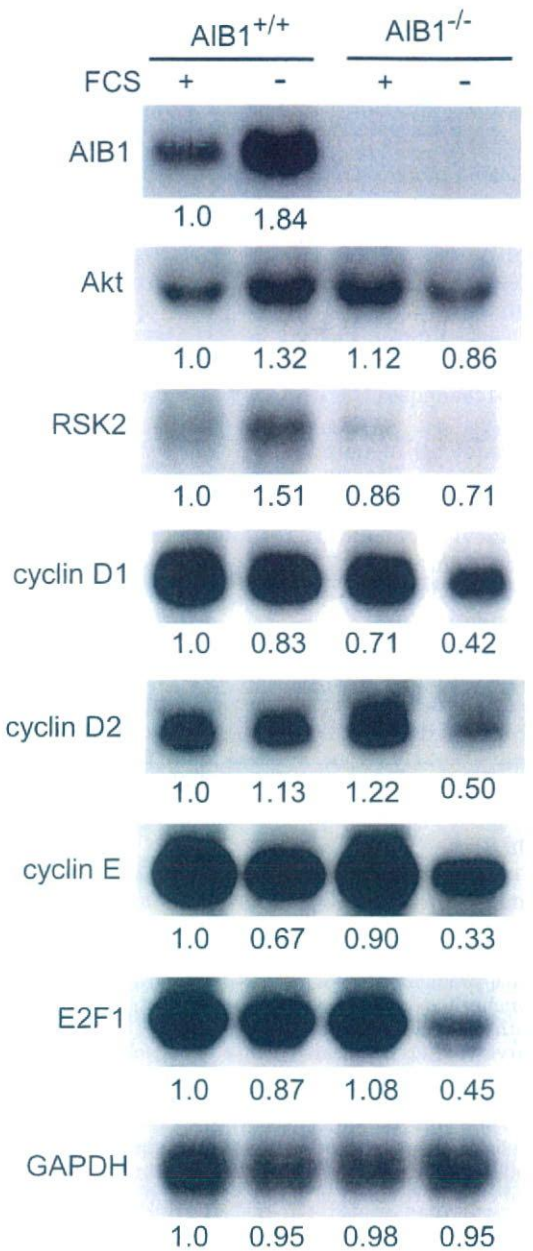


Fig. 4. **Relative levels of expression of mRNAs of cell cycle regulators.** Wild-type and AIB1-knockout DT40 cells were cultured for 8 h in the presence or absence of FCS. Total RNAs were prepared for Northern blot analysis. The radioactivities of the corresponding bands of cell cycle regulators and GAPDH mRNA were determined with an image analyzer as relative intensity, and the normalized intensity against levels of mRNA in wild-type DT40 cells with FCS are shown.

Recent study showed that AIB1 overexpression enhanced the activation of PI3K/Akt pathway and AIB1 knockdown increased apoptosis (*15*). As mentioned above, the stress-activated JNK pathway was suppressed in the presence of AIB1 (Fig. 3B). In order to investigate if the JNK suppression is mediated by the PI3K/Akt pathway, we

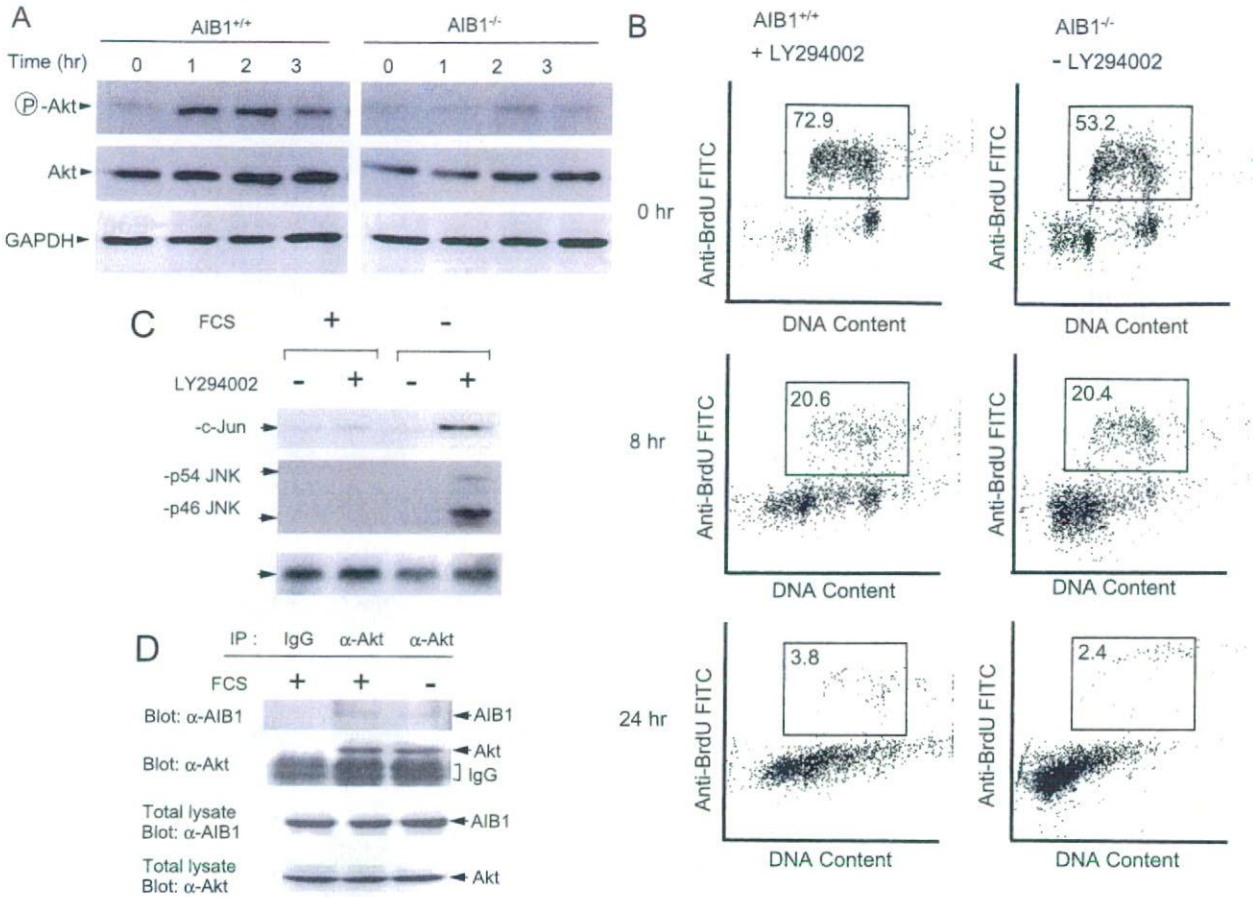


Fig. 5. **Inhibition of Akt or deficiency of AIB1 blocks DNA replication under stress conditions.** (A) Akt phosphorylation and AIB1 production were required for DNA replication. Cells were synchronized for 8 h with hydroxyurea (1 mM), which interfered with cell cycle progression by preventing DNA replication, and were harvested at the indicated points (0–3 h) following release from the cell cycle arrest. Production of phosphorylated Akt and total Akt was determined by immunoblot analysis using anti-phospho-Akt (Ser-473) and anti-Akt antibodies. (B) DNA replication in wild-type DT40 cells by in response to serum deprivation was blocked by LY294002, an inhibitor of PI3K/Akt. Wild-type cells or AIB1-knockout cells were cultured with LY294002 (50 μM) in the absence of FCS for 8 h or 24 h. The percentage BrdU positivity was determined by counting the number of cells at the BrdU-positive gates. (C) The JNK suppression in the AIB1-positive cells was blocked by LY294002. Wild-type cells were cultured with or without LY294002 (50 μM) in the presence or absence of FCS for 8 h. The levels of phosphoprotein in the JNK-mediated apoptosis pathway were determined by Western blot. (D) AIB1 is physically associated with Akt. The DT40 cell extracts were immunoprecipitated with anti-Akt antibody. For control, cell extract was precipitated with the IgG from non-immunized rabbit. The immunoprecipitates (IP) were subjected to Western blot analysis with the indicated antibodies.

examined the phosphorylation of c-Jun and JNK after LY294002 treatment. As a result, we found the marked increase of phospho-c-Jun and phospho-JNK by the LY294002 treatment in response to serum starvation (Fig. 5C), suggesting that the inhibition of PI3K/Akt pathway blocked the AIB1-mediated JNK suppression. Further, we showed the direct interaction between AIB1 and Akt (Fig. 5D). Collectively, these data suggested that AIB1 might be activated by Akt.

Loss of AIB1 Leads to Inhibition of UV-Induced Phosphorylation of Histone H3 at Serine 10—DNA replication is linked to chromatin modulation. This is associated with the modification of chromatin-associated proteins such as histones (H2A, H2B, H3, and H4) or remodeling cofactors, which are known to possess intrinsic histone acetyltransferase activity and are capable of chromatin modification by histone acetylation (*9*, *10*). We have shown here that AIB1 depletion causes a marked defect in the ability to induce DNA replication in response to cellular stress. Therefore, AIB1 may control the signal cascades for the remodeling of chromatin in response to cellular stress. We next examined whether AIB1 was required for phosphorylation of chromatin modulation in response to cellular stress. By Western blot analyses using cell extracts of acid-soluble proteins, we found that wild-type cells had a marked increase in the phosphorylation of histone H3 at serine 10 (Fig. 6), but not in the acetylation of histone H3 at lysine 9 or 14 (data not shown), very soon after treatment with UV-irradiation. This phosphorylation was completely eliminated under stress conditions by the loss of AIB1 in AIB1-knockout cells (Fig. 6). Although a previous study had shown that AIB1 possesses intrinsic histone acetyltransferase (HAT) activity for chromatin modification (*2*), we observed no difference in the levels of acetylation of histone H3 at lysine 9 and lysine 14 in AIB1-knockout DT40 cells compared with wild-type cells

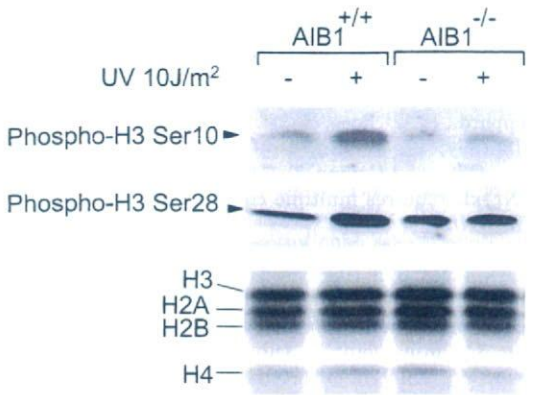


Fig. 6. **Knockout of AIB1 leads to inhibition of UV-induced phosphorylation of histone H3 at serine 10.** Western blot analyses are shown with the specific antibodies indicated on the left, in wild-type or AIB1-knockout cells either treated or untreated by UV irradiation (10 J/m^2). Coomassie Blue–stained 15% polyacrylamide SDS–containing gels indicated equal loading of proteins.

following UV irradiation (data not shown). These results suggest that AIB1 plays a critical role in the signaling cascade for both Akt activation and modulation of the phosphorylation of histone H3 at serine 10 in response to cellular stress.

DISCUSSION

Recent studies have demonstrated that AIB1 plays a pivotal role in activation of the intrinsic IGF-1–driven cell survival pathway, which is mediated through the PI3K/Akt pathway (*15*). Although the signal transduction pathways that lead to the positive control of AIB1 have been studied extensively, the critical targets of this kinase that mediate the stress response remain to be determined. We showed here that AIB1-deficient DT40 cells are extremely sensitive to be killed by cellular stresses such as serum deprivation and UV irradiation. This susceptibility was correlated with a reduction in the ability to restore DNA-synthesis levels under stress conditions. Moreover, we showed that, with serum deprivation or UV irradiation treatment, the induction of phosphorylation of both JNK and c-Jun in AIB1-knockout DT40 cells was much greater than in wild-type DT40 cells. These results are consistent with those of previous studies, which demonstrated that activation of the JNK/c-Jun pathway mediates the induction of cell death by DNA damage agents (*33*, *34*). Our results therefore indicate that the presence of AIB1 is required to suppress activation of the JNK/c-Jun signaling pathway in DNA replication under cellular stress conditions. On the other hand, in ER-negative cancer cells subjected to cellular stress, high levels of AIB1 production have been shown to promote activation of the Akt/p65 survival pathway (*15*). It has been shown that activation of c-Jun by the JNK apoptosis pathway is required to suppress NF-κB transcription (*35*, *36*). Our study is consistent with the result of a recent report, which demonstrated that Akt inhibits stress-activated JNK through activation of NF-κB (*36*). Collectively, these data indicate that AIB1 plays a key role as a mediator between the Akt/NF-κB and JNK/c-Jun pathways in controlling cell fate in response to cellular stress.

Importantly, various signal transduction pathways can modulate the interactions of specific coregulators with nuclear receptors or mediate their activities (*37*). Recent studies suggest that the transcriptional corepressors NCoR and SMRT interact with, and exert repressive effects on AP-1 or NF-κB (*38–41*). It has been proposed that the transactivation potential of c-Jun is repressed by histone deacetylase (HDAC) complexes and these repressor complexes are dissociated by JNK-mediated phosphorylation (*42*). As can be seen from our stress assays in DT40 cells, presence of AIB1 is an important key to modulate the switch from transcriptional repression to activation in association with the diverse protein kinase-dependent signalling pathways in response to cellular stress.

We have shown that, in addition to its role in signal ransduction pathways, AIB1 production is correlated with UV-induced phosphorylation of histone H3 at serine 10, but not with acetylation of histone H3 at lysine 9 or 14. These results suggest that AIB1 is essential in mediation of the phosphorylation of histone H3 in chromatin remodeling. Although it has been shown that the phosphorylation of histone H3 is mediated by the Aurora B kinases (*43*), IKK-*a* (*45*), MSK1 and MSK2 (*45*), and RSK-2 (*46*), we need to reveal by future studies a kinase that can phosphorylate histone H3 in association with AIB1 directly. Previous studies have reported that IκB kinase, a positive regulator of NF-κB activation, is activated by Akt (*13*, *14*). However, production of endogenous IKKα or IKKβ proteins did not change detectably in response to activated Akt in DT40 cells (data not shown). NF-κB activation by stress stimuli has also been shown to be independent of phosphorylation of IκBα at Ser 32/36, and to be IKK-independent (*47*). Moreover, NF-κB, which is usually maintained in an inactive state by protein–protein interaction with inhibitor IκBs, is constitutively active in ER-negative breast cancer cell lines (*48*). Thus, in DT40 cells AIB1 might be produced as one of the downstream targets interacting directly with Akt, independently of IKK.

Previous studies have found that, under conditions of stress, Akt interacts with JIP1 in primary neurons and thus inhibits JNK activation. Therefore, ectopic expression of Akt attenuated stress-induced apoptosis while Akt1 gene deletion rendered neurons more sensitive to stress stimulus than wild-type neurons (*49*). Moreover, recent studies have shown that activation of NF-κB is required for inhibition of JNK in response to TNF-α or UV stimulation (*21–24*). These previous studies and our findings suggest that the level of production of AIB1 is a key determinant of cell susceptibility to cellular stress, in association with phosphorylation cascades. Furthermore, we have elucidated the molecular mechanisms by which, in response to cellular stress, AIB1 plays a critical role in DNA replication or phosphorylation of histone H3 at serine 10, in association with active Akt/NF-κB pathway.

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