Communication

Phosphorylation of Bcl-2 Protein and Association with p21\textsuperscript{Ras} in Ras-induced Apoptosis\textsuperscript{*}

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Ras-induced apoptosis can be blocked by the proto-oncogene bcl-2, but the biochemical or functional nature of Bcl-2 regulation of Ras-induced apoptosis is not understood. We demonstrate that Bcl-2 and p21\textsuperscript{Ras} molecules may be co-immunoprecipitated in Jurkat cells. The level of this association is enhanced when an apoptotic stimulus (inhibition of PKC activity) is delivered. Bcl-2/p21\textsuperscript{Ras} association is coincident with new phosphorylation of the Bcl-2 protein. Inhibition of this phosphorylation prevents protection from apoptosis by Bcl-2, providing a functional correlation to the phosphorylation event. The Bcl-2/p21\textsuperscript{Ras} association cannot be competed by exogenous glutathione S-transferase-Ras fusion protein, suggesting that the endogenous complex may be formed before cell lysis. These results provide a possible mechanism of regulation of Ras-induced apoptosis by Bcl-2.

p21\textsuperscript{Ras}, as a key signal transducer, regulates the proliferation or differentiation of eukaryotic cells (1, 2). The activation state of p21\textsuperscript{Ras} in fibroblasts is determined by the guanine nucleotide exchange factors such as Sos and GDP-releasing factor (3–6). It has also been demonstrated that p21\textsuperscript{Ras} interacts with the serine/threonine kinase Raf-1, which in turn regulates the activity of a kinase cascade that includes MEK and mitogen-activated protein kinases (7–9). In T lymphocytes, p21\textsuperscript{Ras} may be involved in both PKC\textsuperscript{1}-dependent and PKC-independent activation pathways (1).

In addition to its involvement in transducing or promoting cell proliferation or differentiation signals, p21\textsuperscript{Ras} is also thought to be necessary for mediating cell cycle progression, including G\textsubscript{0} to G\textsubscript{1}, and G\textsubscript{2} to M, transitions (11, 12). Recently, it has been reported that Ras may be involved in apoptosis mediated by FAS and tumor necrosis factor receptors (13, 14). Furthermore, we have demonstrated that Ras-generated signals lead not only toward cell growth, but also can initiate apoptosis, depending upon the state of other cellular signaling mediators, such as Bcl-2 and PKC. This Ras-induced apoptosis is triggered after suppression of cellular PKC activity and can be blocked by Bcl-2, a cell survival-promoting factor (15). A proto-oncogene product, Bcl-2, can protect cells from apoptosis induced by certain biological or chemical reagents (16–18). Bcl-2 has been reported to bind to a human Ras-related protein, p23\textsuperscript{Ras} (19), as well as to a downstream effector protein kinase, Raf-1 (20).

Since the structure of p23\textsuperscript{Ras} is highly homologous to p21\textsuperscript{Ras} (19, 20), and because p23 can bind to Bcl-2, we have examined the interactions of these proteins in Jurkat cells co-expressing the proto-oncogene bcl-2 and v-Ha-ras (PH1/bcl-2 cells). It is shown here that overexpressed, activated p21\textsuperscript{Ras} can be co-immunoprecipitated with overexpressed Bcl-2 in PH1/bcl-2 cells, under normal growth conditions. The association of these two molecules becomes more obvious after delivery of an apoptotic signal (down-regulation of PKC) and may be related to a post-translational modification of Bcl-2, phosphorylation, which occurs simultaneously. Cell fractionation experiments suggest that the binding of these molecules may take place in the cell membrane. The interaction of Bcl-2 and p21\textsuperscript{Ras} may be likely to be of physiological and biochemical relevance in the regulation of the Ras-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

Cell Transfections—Jurkat cells were stably co-transfected with v-Ha-ras expression vector and a vector containing a selectable marker conferring resistance to Genetin by electroporation as described previously (21). After transfection, these cells (designated PH1) were carried in growth medium plus 0.7 mg of Genetin (Life Technologies, Inc.) per ml. The bcl-2 retroviral expression vector was used to infect either Jurkat or PH1 cells in the presence of Polybrene (Sigma) at 8 μg/ml, as described previously (21). After infection, cells which had stably integrated the vectors were selected in the growth medium containing 200 μg of hygromycin/ml.

Immunoprecipitation and Western Blot Analysis—Cells (2 × 10\textsuperscript{6}), with or without PMA (500 nm) treatment for 24 h, were washed with 1 × phosphate-buffered saline twice and lysed in 1% Triton X-114 lysis buffer (22). The lysate was adjusted to 0.4 M NaCl, 0.5% deoxycholate, and 0.05% sodium dodecyl sulfate (SDS). For each sample, a duplicate cell lysate preparation was made for reciprocal immunoprecipitation and immunoblotting. Two hundred μg of cell lysate was subjected to immunoprecipitation by using either anti-Ras antibody (Signal Transduction Laboratory, Lexington, KY) or anti-human-Bcl-2 antibody (Pharmingen, San Diego, CA). The immunoprecipitates were separated on a 12.5% SDS-polyacrylamide gel. p21\textsuperscript{Ras} or Bcl-2 proteins were detected by immunoblotting with specific antibodies and developed with an anti-mouse Ig alkaline phosphatase reagent (Oncogene Science, Uniondale, NY).

Immunoblot and Kinase Assays—Cells (2 × 10\textsuperscript{6}), under normal growth conditions or after down-regulation of PKC activity, were perma-blized with 1 ml of kinase buffer containing 5 μCi of [\textsuperscript{32}P]ATP for 10 min at 37°C, as described (23). For inhibition experiments, 0.1 μM staurosporine or 200 μM genistein was added to the kinase buffer. Cells were lysed with Triton X-114 lysis buffer (22). Protein extraction and immunoprecipitation of Bcl-2 were performed as described above. After electrophoreses, gels were subjected either to autoradiography directly or to immunoblot analysis and subsequently to autoradiography.

Cell Membrane Fractionation—Cells (2 × 10\textsuperscript{6}), after high dose (500 nm) PMA treatment for 24 h, were resuspended in 0.5 ml of hypotonic buffer (10 mM Tris, pH 7.5, 0.5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride) and incubated at 0°C for 10 min. After centrifugation, the supernatant (cytosolic fraction) and the pellet, resuspended in 100 μl of hypotonic buffer (membrane fraction), were immunoprecipitated by an anti-human-Bcl-2 antibody and immunoblotted by an anti-Ras antibody.
RESULTS AND DISCUSSION

Bcl-2 has been reported to interact physically with R-Ras, which has 55% identity to \( p21^{\text{Ras}} \), or with Raf, which can bind to \( p21^{\text{Ras}} \) and regulate growth signals (19, 20), but little is known about the functional significance, if any, of these interactions. Since the predicted effector domain of \( p23^{\text{Ras}} \) is similar in amino acid sequence to the corresponding region in \( p21^{\text{Ras}} \), the human bcl-2 gene was introduced into J urkast cells (designated as J urkatab-cl-2 cells) and PH1 cells (J urkast cells containing v-Ha-ras, designated as PH1/bcl-2 cells) to explore possible biological and physical interactions between \( p21^{\text{Ras}} \) and Bcl-2. Specific antibodies were used to determine if these proteins could be co-immunoprecipitated, under normal growth conditions or after delivery of a Ras-induced apoptotic signal (down-regulation of PKC activity, by chronic treatment with the phorbol ester PMA) (25–28). We have confirmed elsewhere that this treatment, 24 h of exposure to a high dose (500 nM) of PMA, down-regulates PKC activity in these cells (15).

The proteins co-immunoprecipitating with the anti-\( p21^{\text{Ras}} \) antibody from transfected or control cell lysates, untreated or after PMA treatment, were immunoblotted for Bcl-2 protein using an anti-human Bcl-2 antibody (Fig. 1A). A 26-kDa protein, Bcl-2, was detected in anti-\( p21^{\text{Ras}} \) immunoprecipitates in lysates from PH1/bcl-2 cells, but was not observed in lysates from cells expressing only one of the two transfected genes, or in the control cells. In reciprocal experiments, where the initial immunoprecipitation was carried out with an anti-Bcl-2 antibody, immunoblotting with an anti-\( p21^{\text{Ras}} \) antibody detected a protein of 21 kDa which was co-precipitated in PH1/bcl-2 cells, both under normal growth conditions and after down-regulation of PKC activity (Fig. 1B). The amount of the co-precipitating protein after down-regulation of PKC activity by PMA treatment for 24 h was increased in both cases (a 2.3-fold increase in Bcl-2 protein associating with \( p21^{\text{Ras}} \) and a 1.8-fold increase in \( p21^{\text{Ras}} \) associating with Bcl-2, as measured by densitometry). As a control, an unrelated IgG isotype mouse monoclonal antibody was used for immunoprecipitation, followed by immunoblotting with anti-Bcl-2 or anti-Cod to exclude nonspecific binding. There was no co-immunoprecipitation of either Bcl-2 or \( p21^{\text{Ras}} \) with this irrelevant, isotype-matched IgG control antibody (data not shown). To further confirm the specificity of the association of Bcl-2 and activated \( p21^{\text{Ras}} \), immunoprecipitation with anti-human Bcl-2 antibody was performed after \( ^{35} \)S-methionine/cysteine metabolic labeling of transfected and control Jurkat cells, following down-regulation of PKC activity. 26-kDa (Bcl-2) and 21-kDa (\( p21^{\text{Ras}} \)) species were co-precipitated from the lysates of PH1/bcl-2 cells, but not from the control Jurkat cells (Fig. 1C). In contrast, only the 26-kDa Bcl-2 protein was precipitated from lysates of J urkatab-cl-2 cells. These results confirmed that activated \( p21^{\text{Ras}} \) and Bcl-2 are able to associate physically in vitro and perhaps in vivo. The quantitative increase in association of the two molecules after down-regulation of PKC was not due to changes in the levels of the Bcl-2 protein. The 26-kDa Bcl-2 protein was detected readily by immunoblotting lysates from the cells transfected with the bcl-2 gene, both under normal growth conditions and after down-regulation of PKC, and there was no significant change in Bcl-2 levels under either circumstance (Fig. 1D). In other studies, we have found that delivery of a different signal for apoptosis, stimulation through FAS/APO-1, also results in dramatic enhancement of the association of Bcl-2 and \( p21^{\text{Ras}} \).

It has been suggested that the Bcl-2 protein requires post-translational modification, specifically phosphorylation, for function (29, 30). To determine whether the increased binding of Bcl-2 to \( p21^{\text{Ras}} \) following suppression of PKC activity might be related to a modification of Bcl-2, the phosphorylation state of Bcl-2 in vitro was studied. New phosphorylation of the 26-kDa Bcl-2 protein at increased levels was detected after down-regulation of PKC activity in PH1/bcl-2 cells which had been preloaded with \( ^{32} \)P]ATP (Fig. 2A, lane 5). Bcl-2 was not newly phosphorylated in J urkatab-cl-2 cells under these same conditions.

In normal lymphocytes and cell lines like J urkast, down-regulation of PKC activity can comprise a normal physiologic signal to inhibit cell growth, rather than inducing apoptosis (25–27, 31, 33, 34). Like parental J urkast cells, J urkatab-cl-2 cells only arrest in the G1 phase of the cell cycle after down-regulation of PKC and re-enter the cell cycle when this inhibition is relieved (15). Thus, phosphorylation of Bcl-2 appears to occur only in cells for which inhibition of PKC would induce a cell death program (i.e. cells containing activated \( p21^{\text{Ras}} \), PH1). In similar studies, using metabolic labeling of cells with \( ^{32} \)P]orthophosphate, Bcl-2 was again found to be phosphorylated exclusively in PH1/bcl-2 cells and only during stimulation of Ras-induced apoptosis by inhibition of PKC activity (15). Immuno-
precipitation using an anti-Bcl-2 antibody after \(^{32}\text{P}]\text{ATP}\) labeling, followed by simultaneous autoradiography and immunoblotting, was performed to confirm the identity of the phosphorylated 26-kDa band as Bcl-2. The labeled 26-kDa protein was immunoreactive with the Bcl-2 antibody (Fig. 2B, lanes 1 and 3). Another newly kinase protein species of approximately 20 kDa, which co-precipitated with Bcl-2, was also detected, and may possibly represent the Bcl-2 partner, Bax, although its identity has not yet been confirmed. Phosphorylation of both of these proteins was inhibited by the serine/threonine kinase inhibitor, staurosporine, which suggested the involvement of a serine/threonine kinase in Bcl-2 phosphorylation during stimulation of Ras-induced apoptosis (Fig. 2A). In contrast, the tyrosine-protein kinase inhibitor genistein had no effect on the phosphorylation of Bcl-2 in this permeabilized cell system (data not shown). If phosphorylation of Bcl-2 were required for protection from Ras-induced apoptosis, serine/threonine kinase inhibitors would be expected to abrogate this protective effect. Inhibition of cellular PKC activity by chronic treatment with PMA or by treatment with staurosporine led to apoptosis in cells containing activated p21Ras, as expected (Table I), but protection by Bcl-2 (PH1/bcl-2 cells), as seen in cells treated with PMA, did not occur in the presence of staurosporine.

**Fig. 2. Immunoprecipitation and kinase assays.** A, cells were permeabilized under normal growth conditions (lanes 1–4), after down-regulation of PKC activity (lanes 5–8), or in the presence of staurosporine after down-regulation of PKC (lanes 9–12), and labeled with \(^{32}\text{P}]\text{ATP}\). Subsequently, the labeled proteins were immunoprecipitated by an anti-human Bcl-2 antibody. Lanes 1, 5, and 9, PH1/bcl-2 cells; lanes 2, 6, and 10, PH1 cells; lanes 3, 7, and 11, J urkat/bcl-2 cells; lanes 4, 8, and 12, J urkat cells. B, immunoblotting of labeled proteins. After in vitro phosphorylation and immunoprecipitation, the phosphorylated proteins from PH1/bcl-2 or PH1 cells were immunoblotted with an anti-human Bcl-2 antibody. Lanes 1 and 2, autoradiograms of immunoprecipitated proteins; lanes 3 and 4: immunoblots of the same membranes used for autoradiography in lanes 1 and 2. Lanes 1 and 3, PH1/bcl-2 cells; lanes 2 and 4, PH1 cells.

**Table 1**

| Line | Control | PMA × 24 h | Staurosporine |
|------|---------|------------|---------------|
|      | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 |
| Day 2 | 0.65 | 0.50 | 0.55 | 0.53 | 0.26 | 0.25 | 0.12 | 0.24 | 0.25 | 0.31 | 0.15 | 0.14 |
|      | (0.037) | (0.033) | (0.043) | (0.054) | (0.017) | (0.04) | (0.025) | (0.014) | (0.014) | (0.005) | (0.037) | (0.037) |
| Day 3 | 1.10 | 0.90 | 1.05 | 0.95 | 0.35 | 0.32 | 0.10 | 0.28 | 0.44 | 0.52 | 0.10 | 0.10 |
|      | (0.044) | (0.043) | (0.04) | (0.056) | (0.06) | (0.04) | (0.00) | (0.047) | (0.033) | (0.023) | (0.016) | (0.024) |

*a* Cell lines: 1, Jurkat cells; 2, Jurkat/bcl-2 cells; 3, PH1 cells; 4, PH1/bcl-2 cells.

*b* 0.25 × 10⁶ cells/ml seeded on Day 0.

*c* 0.25 × 10⁶ cells/ml seeded on Day 0, exposed to 500 nM PMA for 24 h.

*d* 0.25 × 10⁶ cells/ml seeded on Day 0, exposed to 0.1 μM staurosporine.

*e* Trypan blue excluding cells (× 10⁶) were enumerated in quintuplicate on days 2 and 3. Values (± S.D.) were averaged over three independent experiments.

It is possible that this post-translational modification of Bcl-2 (phosphorylation) occurs only when cells are facing a stress or death challenge. Whether this phosphorylation of Bcl-2 regulates its survival-promoting activity, and whether this modification is the direct result of down-regulation of PKC, or instead is the result of the activation of a rescue pathway in the face of impending apoptosis remains is not yet clear (15). In support of a role for phosphorylation of Bcl-2 in promoting survival in these cells is our finding that, whereas overexpressed Bcl-2 protects PH1 cells from undergoing apoptosis during down-regulation of PKC, it does not protect these same cells from apoptosis during inhibition of PKC by staurosporine, a general serine/threonine kinase inhibitor. We have demonstrated herein that the serine/threonine kinase responsible for phosphorylating Bcl-2 under these conditions is sensitive to staurosporine, suggesting a requirement for this post-translational modification of Bcl-2 for its protective function. When a more specific inhibitor of PKC, chelerythrine, was utilized in parallel experiments, chelerythrine was also found to activate apoptosis in PH1 cells and with faster kinetics than did down-regulation of PKC by chronic exposure to PMA, as might be expected from the more rapid action of chelerythrine on inhibition of PKC activity. Yet, protection from Ras apoptosis by Bcl-2 was maintained, in contrast to the findings with staurosporine, and phosphorylation of Bcl-2 in response to apoptotic stimuli was not inhibited (15).

These results suggested that p21Ras and Bcl-2 can interact within cells, under conditions where the bcl-2 gene is overexpressed, and this binding becomes prominent in response to activation of the Ras-induced apoptosis program. To determine whether the formation of this complex occurred in whole cells, or after lysis of the cells, a GST-Ras fusion protein bound to agarose was added to the cell lysates, and the precipitates were immunoblotted with anti-human Bcl-2 and anti-p21Ras antibodies. A 26-kDa Bcl-2 band was detected only in lysates from J urkat/bcl-2 cells (Fig. 3A). Thus, a direct interaction of p21Ras and Bcl-2 could be demonstrated in vitro. In the PH1/bcl-2 cells, however, no such interaction could be detected. This suggests that the complex of endogenous p21Ras and Bcl-2 is formed before lysis of the cells, and, that once formed in vivo, the association of these two molecules could not readily be competed by addition of exogenous GST-Ras to the cell lysates for 2 h. In the absence of activated p21Ras, inhibition of PKC activity itself does not cause apoptosis in J urkat cells (Table I and Ref. 15), and no new phosphorylation of Bcl-2 protein, even when overexpressed, was detectable (Fig. 2). The inability of GST-Ras to compete for Bcl-2 in the PH1/bcl-2 cells lysates over 2 h may therefore reflect a higher affinity of phosphorylated Bcl-2 for p21Ras in general, or for activated p21Ras in particular. To demonstrate that competition for Bcl-2 could occur as a function of time or concentration of the competitor (GST-Ras), the length of time of incubation or the concentration of GST-
Bcl-2 Phosphorylation and Association with p21Ras

In our studies, p21Ras is in a constitutively activated (GTP-bound) state. The p21Ras/Bcl-2 association was most prominent under conditions favoring apoptosis in v-ras-expressing cells. Finally, the associations we demonstrate may require post-translational modification of Bcl-2 and Ras (30, 39, 40), which would not be observed in a two-hybrid system. Membrane localization of p21Ras with p21Ras has been implicated as a mediator of diverse inducers of apoptosis (13–15). The interaction of Bcl-2 and p21Ras may thus be of general physiological and biochemical relevance in the regulation of apoptosis by Bcl-2.

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