

A Role for Jun-N-Terminal Kinase in Anoikis; suppression by bcl-2 and crmA

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Abstract. The disruption of interactions between extracellular matrix and specific cognate integrins triggers apoptosis in epithelial cells, in a process termed "anoikis." To understand anoikis, the connections between epithelial cell integrin signaling and the apoptosis-regulatory proteins are being explored. We report herein that early after detachment from matrix, epithelial cells activate Jun-N-Terminal Kinases (JNKs; alternatively known as Stress-activated Protein Kinases), which are also activated by other apoptotic stimuli. The activity of this pathway was required for anoikis. Another early response to cell suspension was the activation of the ICE-related cysteine protease, ICE/LAP3; this activation and anoikis were suppressed by the ICE-

protease inhibitor, crmA. The overexpression of bcl-2 suppressed ICE/LAP3 activation as well. Surprisingly, bcl-2 and crmA attenuated the activation of JNKs following cell suspension, suggesting that the JNK pathway is regulated directly or indirectly by proteolysis. In addition, the blockage of the JNK pathway attenuated the activation of ICE/LAP3, suggesting a positive feedback loop between the ICE and JNK systems. These results indicate the following sequence of information flow in anoikis: integrins→bcl-2/bax→(ICE-proteases↔JNK)→apoptosis. Cell-cell interactions, which were previously shown to sensitize cells to anoikis, caused bcl-2 mRNA to be downregulated, a permissive event for downstream apoptotic signaling.

THE acquisition of anchorage independence (Shin et al., 1975) and apoptosis resistance (Thompson, 1995; Kerr et al., 1994; Fisher, 1994; Reed, 1995) are critical for tumor malignancy. The concept of anoikis (for review see Ruoslahti and Reed, 1994) explains how apoptosis resistance can lead to anchorage independence of epithelial (Frisch, Francis, 1994) and endothelial (Meredith et al., 1993) cells.

An understanding of anoikis will require the elucidation of the mechanisms for integrin signaling in epithelial cells and how these connect with apoptosis-regulatory proteins. Cell shape, regulated by integrin-matrix interactions, is an important link between integrins and apoptosis (Re et al., 1994) that can be considered one form of integrin signaling. However, the inappropriate activation of certain integrin signaling molecules can bypass the cell shape requirement for the suppression of apoptosis, as it occurs in oncogenic transformation. For example, we have recently shown that an experimentally activated form of focal adhesion kinase (FAK)¹ can render certain epithelial cells anoikis-resistant and transformed (Frisch et al., 1996).

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1. *Abbreviations used in this paper:* FAK, focal adhesion kinase; JNK, Jun-N-terminal kinase.

Conversely, a tumor suppressor gene that transcriptionally programs an epithelial phenotype (E1a) renders human carcinoma or sarcoma cells anoikis-sensitive and non-transformed (Frisch, 1994; Frisch, 1991; Frisch and Dolter, 1995).

Studies of integrin signaling in fibroblasts have indicated that cell-matrix adhesion causes a transient activation of the ERK1,2 enzymes of the MAP kinase family (Chen et al., 1994). This has been proposed to occur via the binding of FAK to *c-src* family proteins (Cobb et al., 1994) and/or nucleotide-exchange factors for ras (Schlaepfer et al., 1994). In an epithelial cell system, however, the activation of ERK1,2 in response to cell-matrix interaction was not evident, nor was it enhanced by expression of an activated form of FAK (Frisch et al., 1996). This motivated us to examine alternative protein kinase signaling events that might relate to anoikis.

JNKs (alternatively known as SAPK, Stress-activated Protein Kinases) have previously been shown to be activated by various apoptotic stimuli such as ultraviolet light, interleukin-1, tumor necrosis factor- α , and γ -irradiation (Davis, 1994; Derijard et al., 1994; Verheij et al., 1996). In the present study, we report that JNKs are also activated by the disruption of integrin-mediated cell-matrix interactions, and that this activation is required for anoikis. We further show that bcl-2 and crmA attenuate the JNK activation, which functionally links the bcl-2/bax, ICE, and JNK pathways.

Materials and Methods

Construction of Cell Lines

The wild-type and dominant-negative (Whitmarsh et al., 1995) FLAG-epitope-tagged JNKK coding sequences were provided by R. Davis, University of Massachusetts; bcl-2 wild-type and point mutant 145 coding sequences (Hanada et al., 1994) were provided by J. Reed (the Burnham Institute, La Jolla, CA); crmA wild-type and crmA point mutant 291 coding sequences (Tewari et al., 1995), provided by G. Salvesen, the Burnham Institute were subcloned into the retrovirus vector pBABEpuro (Morgens-tern and Land, 1990), provided by H. Land and packaged amphotropically in Bing cells (American Type Culture Collection, Rockville, MD) as described (Pear et al., 1993). 2 d after transfection, viral supernatants were applied to MDCK cells, which were then selected for 10 d in 1.75 μ g/ml puromycin. Individual colonies were picked, expanded, and tested for expression of the transgene by Western blotting. For all experiments reported here, two to three independently derived cell lines of each type were tested at least twice; however, for brevity, only one representative result is shown. The ras(val12)-transformed MDCK cells were described previously (Frisch and Francis, 1994).

Western Blot Analysis

Western blots were performed as described previously (Frisch, 1994) using antibodies from the following sources: anti-FLAG M2 (IBI-Kodak, Rochester, NY); anti-bcl-2 COOH terminus and anti-JNK (Santa Cruz Biotechnology, Santa Cruz, CA); anti-crmA (D. Pickup, Duke University); and anti-ICE/LAP3 (V. Dixit, University of Michigan).

JNK Assays

JNK activity was assayed as described (Derijard et al., 1994) using the "pull-down" method wherein GST-jun fusion protein is used to affinity-select JNK enzyme before the kinase reaction. Cells were not serum starved before induction by cell suspension. Within each experiment, an equal amount of protein (determined by Bio-Rad protein assays) was assayed for each sample. Quantitative results were obtained using a GS250 phosphorimager (Bio-Rad Labs, Hercules, CA). For the immune complex kinase assay (Derijard et al., 1994) of JNK, anti-JNK antibody (Santa Cruz Biotechnology) was used.

Anoikis Assays

Cells were assayed for anoikis by agarose gel analysis of internucleosomal DNA cleavage as described previously (Frisch and Francis, 1994), except that the assay used 0.5×10^6 cells suspended in 2.0 ml of growth medium in a microfuge tube for 3.5 h with slow rotation at 37°C. The loading volumes for the DNA ladder analysis were normalized against the amount of Triton-soluble protein present in the sample before phenol extraction.

RT-PCR Analysis

RT-PCR was performed as described (Ausubel et al., 1994) using the following primers: TGCCACCTGTGGTCCACCTGACCCTC (bcl-2 forward); TTCAGAGACAGCCAGGAGAAATC (bcl-2 reverse); CAGGATGC-GTCCACCAAGAAGCTG (bax forward); TCAGCCCATCTTCTC-CAGATGG (bax reverse).

Results

Suspension of MDCK Cells Induces JNK Activity

We showed previously that 3–5 h of cell suspension irreversibly committed MDCK cells to anoikis (Frisch and Francis, 1994). Various methods of detachment (EDTA, trypsin, and RGD peptides) produced similar results; for convenience, trypsinization was used in this study. We also showed that cell–cell interactions sensitized cells to anoikis; thus, cells became apoptotic after suspension only if they were confluent before suspension.

Jun-N-terminal kinase (JNK) activity was assayed in

cells that were grown to subconfluence or confluence and subsequently placed in suspension. In parallel with the anoikis response, JNK activity was rapidly and strongly induced by the suspension of confluent cells but not subconfluent cells (Fig. 1 A). This result indicated that JNK is induced by the disruption of cell–matrix interactions, and that this effect is modulated by cell–cell interactions, suggesting a possible role for JNK in anoikis.

While the substrate that we used for the JNK assays, c-jun amino acids 1–223, contains residues specifically phosphorylated by JNK (Derijard et al., 1994), it was for-

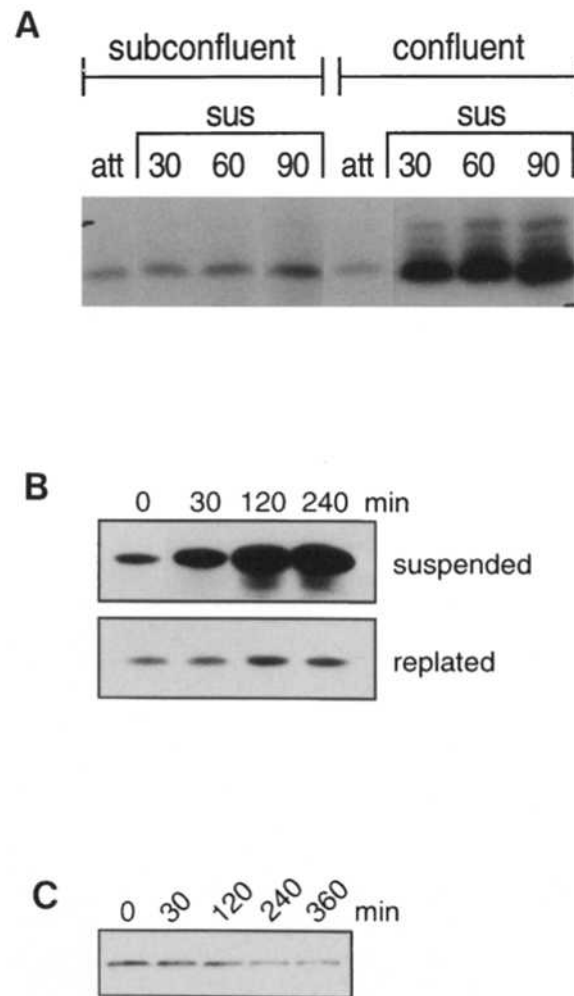


Figure 1. JNK activity is induced by the disruption of cell–matrix interactions (cell suspension). (A) Cell–cell interactions sensitize JNK to being activated by disruption of cell–matrix interactions. MDCK cells grown to confluence or subconfluence ($\sim 30\%$ of confluent cell density) were assayed for JNK activity, using the pull-down assay described in Materials and Methods, either under attached conditions (*att*) or after cell suspension for the times indicated (*min*). (B) The jun-kinase activity induced by cell suspension is JNK. Immune complex kinase assays using anti-JNK antibody were performed on cells held in suspension for the indicated times. (C) Cell suspension does not induce JNK protein expression. Attached or suspended MDCK cells were lysed and analyzed on a Western blot for JNK expression; a control blot using anti-tubulin antibody demonstrated equal protein loading (data not shown).

mally possible that a different kinase activity was being scored. However, when JNK activity in suspended MDCK cells was assayed alternatively by an immune-complex kinase assay using a JNK(1,2)-specific antibody, similar results were obtained (Fig. 1 B), confirming that the kinase activity was JNK specifically.

When cells were replated on collagen-coated plates (at high density) after trypsinization and neutralization, JNK activity was not induced (Fig. 1 B). This result, and the effect of cell density noted above, indicate that JNK activity is induced by suspension and not by trypsin treatment.

To verify that the induced JNK activity was due to signaling rather than de novo synthesis of new JNK protein, Western blots were performed. The level of JNK protein was neither affected by suspension (Fig. 1 C) nor by cell density (data not shown).

Cell-Cell Interaction Regulates the Expression of *bcl-2* mRNA

Cell-cell interaction before cell suspension sensitizes MDCK cells with respect to anoikis (Frisch and Francis, 1994). Because the ratios of *bcl-2* to *bax* protein in cells are thought to govern their sensitivity to apoptosis (Reed, 1995), the levels of *bcl-2* and *bax* mRNAs were assayed in a non-quantitative fashion by RT-PCR. Interestingly, we found that *bcl-2* mRNA was detectable in subconfluent MDCK cells, which were also resistant to anoikis and refractory to JNK induction. However, confluent MDCK cells—which were sensitive to anoikis and had suspension-inducible JNK activity—had no detectable *bcl-2* mRNA at the level of sensitivity of our assay (Fig. 2). The level of *bax* mRNA, however, remained constant. The canine *bcl-2* and *bax* proteins were not reactive with any anti-human or anti-rodent antibodies tested, prohibiting measurement of the protein levels.

These results indicate the levels of *bcl-2* mRNA were regulated by cell-cell interactions. This decrease correlated with the onset of sensitivity to anoikis and with the sensitivity of JNK to activation by cell suspension.

Bcl-2 Suppresses Suspension-induced JNK Activity

The results above (although purely a correlation between *bcl-2* mRNA levels and JNK activation) suggested that perhaps *bcl-2* regulated the sensitivity of the JNK pathway to induction by cell suspension. To test this possibility, MDCK cells were constructed that overexpressed either the wild-type *bcl-2* protein or a point mutant (*bcl-2*mut145) that is incapable of dimerizing with *bax* or suppressing apoptosis (Hanada et al., 1995). The *bcl-2* (wild-type) cells were substantially resistant to anoikis, while the *bcl-2* (mut145) cells were fully sensitive (Fig. 3). Interestingly, *bcl-2* (wild-type), but not *bcl-2*(mut145), attenuated (~5.6-fold) the induction of JNK activity by cell suspension (Fig. 4 A). Western blot analysis showed that overexpressed *bcl-2* did not affect JNK protein levels (Fig. 4 B), suggesting that *bcl-2* blocks some aspect of the JNK signaling pathway.

Bcl-2 and *crmA* Suppress the Activation of ICE/LAP3

The effect of *bcl-2* on JNK activation motivated experi-

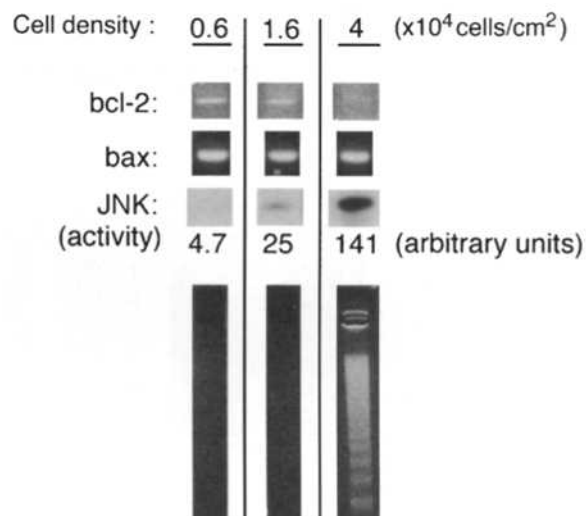


Figure 2. Cell-cell interactions downregulate *bcl-2* mRNA expression. MDCK cells grown in monolayers of low, medium, or high density were analyzed for *bcl-2* and *bax* mRNA expression by RT-PCR reactions on equal amounts of total RNA using the appropriate primers. Cells on parallel dishes grown to each density were trypsinized and placed in suspension for 30 min or 3.5 h, after which protein lysates in JNK extraction buffer or low molecular weight DNA were prepared and assayed for JNK activity or apoptotic DNA ladders, respectively.

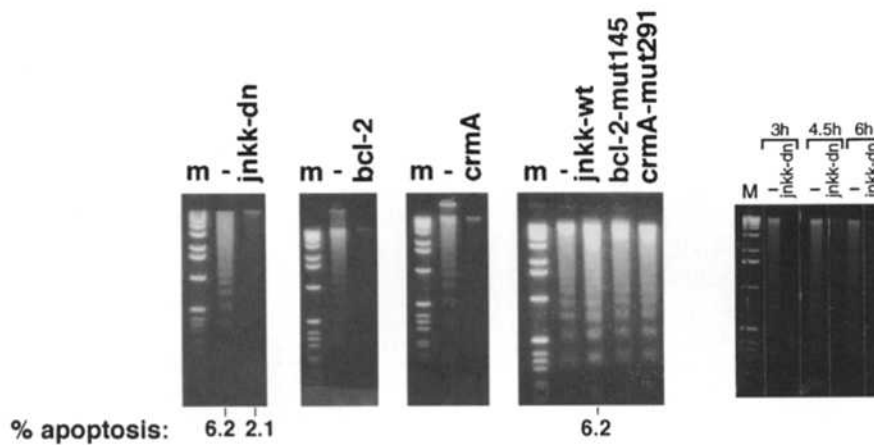
ments to test whether other apoptosis modulatory proteins might affect the JNK pathway as well. The cowpox virus protein, *crmA*, has previously been shown to inhibit apoptosis in many systems, including mammary epithelial cells bound to fibronectin (Boudreau et al., 1995). *CrmA* binds and inhibits cysteine proteases of the ICE family (Tewari et al., 1995a,b). MDCK cells were constructed that expressed either the wild-type form of *crmA* or a point mutant (*crmA*mut291) previously shown to lack protease-inhibitory activity (Tewari et al., 1995a). The *crmA* (wild-type) MDCK cells were substantially resistant to anoikis, while the *crmA* (mut291) cells were fully sensitive (Fig. 3 A).

Previous reports have proposed that *bcl-2* directly or indirectly regulates the activity of the ICE system cascade (Chinnaiyan et al., 1996; Shimizu et al., 1996). To test whether this applied to anoikis as well, MDCK cells expressing ectopic *bcl-2* or *crmA* proteins were placed in suspension. Protein lysates were then analyzed on Western blots for the presence of the activated p20 subunit of the YAMA-like protease, ICE/LAP3 (Duan et al., 1996). As expected, p20 was undetectable in attached cells, but appeared in suspended, control MDCK cells (Fig. 3 B); *crmA*-expressing cells failed to produce p20, presumably because ICE proteases required for ICE/LAP-3 processing were inhibited. *Bcl-2*-overexpressing cells also failed to produce p20, which, consistent with recent reports, suggested that *bcl-2* can suppress the proteolytic activation of YAMA-like proteases.

crmA and Activated *ras* Suppress Suspension-induced JNK Activity

The results above indicated that *bcl-2* inhibited the activation of at least a component of the ICE-like protease cas-

A



B

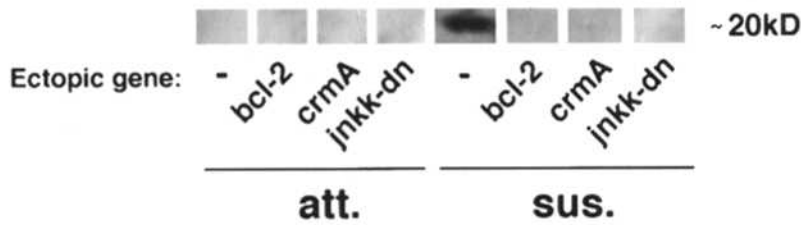


Figure 3. (A) Bcl-2, crmA, and dominant-negative JNKK (*jnkkn-dn*) suppress anoikis. Anoikis assays were performed on stable, retrovirus-transduced cell lines as described in Materials and Methods. The percent apoptosis for three types of cell lines were obtained by flow cytometry of propidium iodide-stained cells (which are likely to represent underestimates). The right-hand panel depicts anoikis assays conducted at later suspension time points. (B) Bcl-2, crmA, and *jnkkn-dn* inhibit the activation of ICE/LAP3. MDCK cells containing the indicated ectopic genes and grown under normal attached conditions (*att*) or held in suspension for 2 h were analyzed by Western blotting using anti-ICE/LAP3 polyclonal antibody. The ICE/LAP3 p20 subunit, resulting from proteolytic activation by upstream proteases, is shown.

cade and inhibited the activation of JNK as well. This suggested that perhaps the ICE-like protease cascade was required for JNK activation.

This possibility was tested using the cell lines described above. Interestingly, crmA (wild-type), but not crmA(mut 291) attenuated (approximately eightfold) the induction of JNK by cell suspension; Western blot analysis (Fig. 4 B) showed that crmA did not affect JNK protein levels.

Transformation of the MDCK cells with an activated form of c-ha-ras (rasV12) both alleviated anoikis and suppressed the activation of JNK, suggesting crosstalk between some component of the ras-raf-MAPK pathway and the JNK pathway.

JNK Mediates Anoikis

Cell suspension induced JNK activity by a bcl-2- and crmA-sensitive mechanism. If JNK were to mediate anoikis, then the suppression of anoikis by bcl-2 and crmA might partly be due to their suppression of JNK. The role of the JNK pathway in anoikis was therefore investigated. A previously described dominant-negative form of JNKK was used, in which serine 257 and threonine 261 were mutated to alanines, thereby preventing the phosphorylation of the enzyme by MEKK that is required for optimal JNKK kinase activity (Whitmarsh et al., 1995). MDCK cell lines were constructed that expressed this mutant (JNKK-dn). These cells had approximately fourfold reduced JNK ac-

tivity after 30 min of suspension, compared with either normal MDCK cells or cells expressing the wild-type JNKK protein (Fig. 4 A). Interestingly, the cells expressing the dominant-negative JNKK were also about threefold more resistant to anoikis when assayed at 3.5 h (Fig. 3 A); substantial anoikis resistance was retained for up to 6 h of cell suspension. Accompanying this, the activation of ICE/LAP3 was substantially attenuated (Fig. 3 B).

Discussion

Integrin Signaling and the JNK Pathway

In this study, the connections between integrin signaling and apoptosis were explored in the context of anoikis regulation. Several previous reports have indicated that the ERK-type MAP kinases are stimulated by integrin-mediated cell adhesion in fibroblasts; this is thought to involve FAK and ras signaling (Chen et al., 1994; Cobb et al., 1994). However, we reported previously that ERKs were not activated in MDCK cells in response to cell-matrix adhesion, even in cells containing an activated form of FAK (Frisch et al., 1996). This motivated us to examine the role of the JNK pathway.

We have now found that JNK is rapidly activated by the disruption of cell-matrix interactions in MDCK cells. The JNK pathway, also known as the Stress-activated Protein

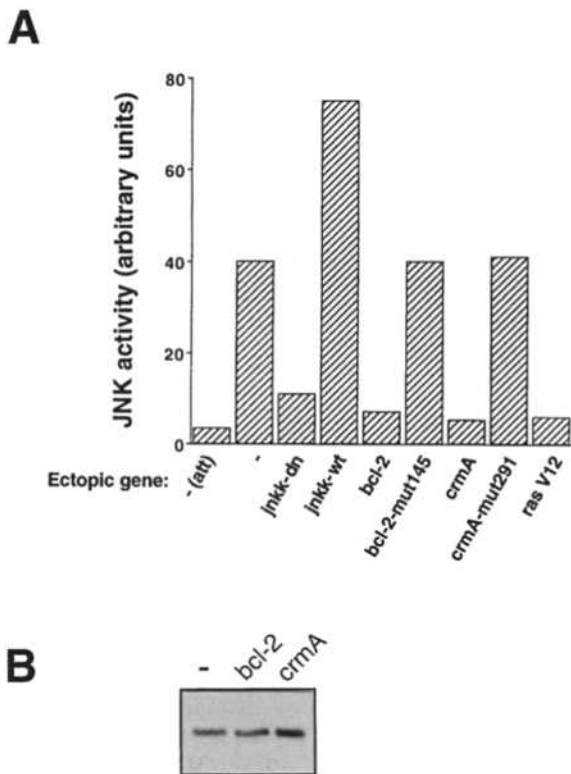


Figure 4. Bcl-2, crmA, JNKK-dominant negative, and c-ha-ras (V12) suppress suspension-induced JNK activity. (A) JNK assays were performed as in Materials and Methods using 150 μ g of cell extract. Cells were assayed after suspension for 30 min, except in attached control (att). (B) Bcl-2 and crmA do not affect the expression of JNK protein. The Western blot using anti-JNK antibody was performed on equal amounts of total cell lysate; a parallel blot using anti-tubulin antibody confirmed equal loading (data not shown).

Kinase pathway, is well known to respond to extracellular stimuli that induce apoptosis, such as tumor necrosis factor- α , ceramide, granzyme B, interleukin-1, ultraviolet light, and γ -irradiation (Davis, 1994; Derijard et al., 1994; Verheij et al., 1996). While this project was in progress, several new reports showed that JNK mediated these apoptotic events. For example, the apoptosis of PC12 cells caused by the withdrawal of nerve growth factor (Lange-Carter et al., 1993) and that of ceramide-treated 3T3 cells (Verheij et al., 1996) were blocked by dominant-negative forms of JNKK. Analogously, we have shown that JNK plays a critical role in mediating anoikis as well. This result demonstrates that integrin-mediated cell-matrix interactions regulate well-characterized stress-response pathway. By contrast, JNK is induced by cell-matrix adhesion in fibroblasts (Miyamoto et al., 1995), underscoring the dramatically different integrin signaling pathways in the two cell types.

The mechanism by which the lack of integrin-matrix interaction triggers JNK activation remains to be elucidated. We have considered several possibilities. The first was that ceramide perhaps would accumulate in detached cells and activate JNK. However, cell suspension was found to induce ceramide accumulation only in subconfluent (i.e.,

anoikis-resistant) cell cultures, rendering improbable an important role of ceramide in anoikis (data not shown). The second possibility was that cdc42 or rac, which can stimulate JNK in transiently transfected fibroblasts (Minden et al., 1995), might be altered by cell adhesion so as to affect the JNK pathway in epithelial cells. However, definitive effects of the activated or dominant-negative forms of these molecules were not observed (data not shown). Other possible mechanisms for connecting integrins with the JNK pathway include changes in cytoskeletal organization.

The mechanisms by which JNK causes apoptosis are presently unclear as well. However, there are two reports that a dominant-negative form of the JNK substrate, c-jun, can confer apoptosis resistance (Lange-Carter et al., 1993; Verheij et al., 1996), suggesting that the latter may function both in apoptosis and transcription.

Connections Among Three Types of Signaling Molecules in Anoikis

Apoptosis is regulated by at least three types of signaling molecules: (1) bcl-2-related proteins (Reed, 1994); (2) ICE-related proteases (Takahashi and Earnshaw, 1996); and (3) JNK pathway kinases (Davis et al., 1994). Recently, it has become clear that ICE-related proteases form a proteolytic cascade (reviewed in Fraser and Evan, 1996). "Initiator" proteases, such as the Fas-associated prototype, FLICE (Muzio et al., 1996), are thought to convert pro-ICE to active ICE. The latter then acts as an "amplifier" protease to convert the pro-forms of the ICE/ced-3 family (e.g., CPP32/YAMA, ICE/LAP-3, etc.) into the active, "machinery" proteases, which degrade substrates such as nuclear lamins and polyADP ribose polymerase. Other reports have shown that the overexpression of bcl-2 or bcl-x_l can prevent the activation of the machinery and/or the amplifier proteases (Chinnaiyan et al., 1996; Shimizu et al., 1996). Consistent with this, we have shown that bcl-2 prevents the activation of the machinery protease ICE/LAP-3; this is presumably one mechanism by which bcl-2 suppresses anoikis.

We have also shown that cell-cell interactions cause bcl-2 expression to be downregulated, sensitizing MDCK cells to subsequent anoikis. After bcl-2 downregulation, proteolytic activation of the ICE-like protease cascade and apoptosis are potentiated; indeed, the protease cascade fails to activate when noninteracting MDCK cells are challenged with an apoptotic stimulus (data not shown). These results underscore the significance of cell-cell interactions in regulating the apoptotic sensitivity of epithelial cells in vivo. They also prompt an examination of the effect of aberrant cadherin-catenin complexes, often found in carcinomas (Birchmeier and Behrens, 1994), upon bcl-2 levels and the ICE system.

Perhaps the most novel result of the present study is that the activity of the JNK pathway requires ICE-system proteolytic function and is therefore inhibited by crmA or bcl-2. These results have several ramifications. First, they functionally link the bcl-2, ICE, and JNK systems into a unified pathway for apoptotic signaling. Secondly, they motivate the search for an ICE-like protease substrate that is proteolytically activated to switch on the JNK pathway; candi-

dates are currently being tested. ICE-system proteases and bcl-2 (or cell-cell interactions) may potentially regulate the phosphorylation and transcriptional activity of JNK kinase-substrates such as c-jun and ATF-2; in this connection, bcl-2 was recently reported to regulate the activity of an NF- κ B kinase (Grimm et al., 1996). Conversely, the dominant-negative form of JNKK attenuated the activation of ICE/LAP3. This indicates that the JNK system is connected with the ICE system via a positive feedback loop that may serve to rapidly amplify the apoptotic response. In the future, it will be of interest to determine the molecular basis of this feedback loop.

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