The Heparin-binding Domain and V Region of Fibronectin Regulate Apoptosis by Suppression of p53 and c-myc in Human Primary Cells*

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Yvonne L. Kapila‡‡, Shaohui Wang‡, Paul Dazin†, Elizabeth Taflola‡, and Marc J. Mass¶

From the ‡Department of Stomatology, School of Dentistry and the †Howard Hughes Medical Institute, School of Medicine, University of California San Francisco, San Francisco, California 94143 and the ¶Environmental Carcinogenesis Division (MD-68), National Health and Environmental Effects Laboratory, United States Environmental Protection Agency, Research Triangle Park, North Carolina 27711

In apoptosis the tumor suppressor p53 and the c-myc proto-oncogene are usually up-regulated. We show a novel alternative pathway of apoptosis in human primary cells that is mediated by transcriptionally dependent decreases in p53 and c-Myc and decreases in p21. This pathway is regulated by the alternatively spliced V region and high-affinity heparin-binding domain of fibronectin. Requirements for c-Myc, p53, and p21 signals in maintaining survival and for their decreases in inducing apoptosis were demonstrated by the ability of p53, c-Myc, and p21 ectopic expression to rescue this apoptotic phenotype, and the ability of p53-deficient and c-myc antisense conditions to trigger a faster rate of apoptosis.

Multiple signaling pathways of apoptosis are triggered by various external insults to or stimuli on the cell. In the case of the extracellular matrix (ECM), it appears that depriving cells of anchorage and/or appropriate survival signals by disrupting the signals mediated by ECM-integrin interactions induces a pathway of apoptosis for which some signaling components have been identified (1, 2). This pathway seems to be initiated with the loss of phosphorylation of the integrin-associated signaling molecule focal adhesion kinase (pp125FAK) (1–3).

The downstream signals to which pp125FAK has been linked include the caspase family of cell death proteases. Caspases, which are considered the executioners of cell death in apoptosis, are thought to orchestrate cell disintegration through a cascade of caspase activation. This family of aspartate proteases seems to be involved in apoptotic pathways regulated by the ECM, because caspase inhibitors prevent apoptosis triggered by an altered matrix, loss of the ECM, or disruption of pp125FAK function (2–4). Furthermore, caspase 3 has recently been shown to cleave pp125FAK as part of the mechanism that leads to apoptosis (5, 6).

Other signals implicated in apoptosis triggered by an altered matrix or disruption of ECM-integrin signaling pathways include the tumor suppressor p53 and the oncogene c-myc, which are known mediators of apoptosis in other pathways (7). In general, activation of p53 and c-Myc corresponds to the apoptotic phenotype, and conversely, inactivation of p53 and c-Myc corresponds to cell survival. In the case of apoptosis regulated by the ECM, p53 may either play a direct role (3) or only modulate the kinetics of this pathway (8). c-Myc, which in some apoptotic pathways modulates p53 function, is involved in apoptosis triggered by disruption of integrin signals, because during c-Myc-induced apoptosis there is targeted proteolysis of pp125FAK, which can be suppressed by integrin signaling (9). The protein encoded by the p53 response gene, the inhibitor of cyclin-dependent kinases, p21 (10), has also been implicated in integrin-ECM-mediated apoptosis.

However, we now document an interesting and new alternative pathway of apoptosis that is regulated by the alternatively spliced V region and the heparin-binding domain of fibronectin (FN) and leads to transcriptionally dependent decreases in p53 and c-Myc in primary, nontransformed cells. Furthermore, the decreases in p53 and c-Myc are in part driving this mechanism, because transfection with either p53 or c-myc rescues the apoptotic phenotype, and p53-deficient and c-myc antisense conditions trigger a faster rate of apoptosis.

EXPERIMENTAL PROCEDURES

Fibroblast Cell Culture and Plating—Primary cultures of human fibroblasts were obtained, cultured, and tested for apoptosis as previously described (11). P53-deficient and wild-type mouse fibroblasts were provided by Dr. Caroline Damsky (1) and maintained in culture medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin/fungizone) prior to experimentation.

Recombinant FN Proteins—Four recombinant FN protein fragments were tested in these experiments. These fragments, described elsewhere (2), either included (V+) or excluded (V−) the alternatively spliced V region and contained either an unmutated (H+) or a mutated, nonfunctional, high-affinity, heparin-binding domain (H−).

Western Blot Analysis—For Western blot analysis, cells were incubated with the V+ fragment, the V− fragment, or control serum-free medium as indicated for each figure. After incubation, cell lysates were prepared using 100 μg/well TNE buffer (1% Nonidet P-40, 10% glycerol, 150 mM sodium chloride in Tris, pH 7.4, and 1 mM EDTA) containing various protease inhibitors (1 mM sodium orthovanadate, 50 μM sodium molybdate, 25 μg/ml aprotinin, 25 μg/ml leupeptin, 1 mM sodium fluoride, and 1 mM phenylmethylsulfonyl fluoride). Lysates were adjusted for protein concentration using the BCA protein assay kit (Pierce) and then analyzed by standard SDS-PAGE. After electrophoresis, the gels were transferred to nitrocellulose by standard methods.

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† To whom correspondence should be addressed: Box 0512, University of California San Francisco, 513 Parnassus Ave., San Francisco, CA 94143-0512. Tel.: 415-502-4591; Fax: 415-502-4990; E-mail: ykapila@itsa.ucsf.edu.

‡ The abbreviations used are: ECM, extracellular matrix; FN, fibronectin; V+H and V−H, fibronectin fragments containing the alternatively spliced V region and a mutated or wild-type heparin-binding domain, respectively; GAPDH, glutaraldehyde-3-phosphate dehydrogenase; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus.
Fibronectin Apoptosis by Suppression of p53 and c-myc

FIG. 1. Induction of apoptosis by the V^+H^— FN fragment in human primary fibroblasts and concomitant decrease in c-Myc, p53, p21, and bcl-2. a, cell shape changes associated with the recombinant FN proteins. Primary human fibroblasts were allowed to spread for ~2 h and were then incubated with control serum-free medium (CTL) or medium supplemented with the V^+H^—, the V^+H^+, the V^+H^−, or the V^+H^− protein as described previously (11). b, nuclear morphology of 4,6-diamidino-2-phenylindole-stained cells incubated with the recombinant FN proteins described as in a. Photographs in panels a and b were taken after 7 h of treatment. c, DNA fragmentation in cells incubated with the recombinant FN proteins for 14 h. Both cell lysates and supernatants were evaluated in the enzyme-linked immunosorbent assay (11). Fragment levels are represented as optical density (mean OD) units; values represent means and S.D. for three experiments. d, Western blot analysis of cells incubated with V^+H^− or V^+H^+ fragments or control medium for 3 and 7 h. Cell lysates were adjusted for protein concentration prior to analysis. The primary antibodies used to detect the respective proteins included a mouse anti-human c-Myc, mouse anti-human p53, mouse anti-human p21, and mouse anti-human Bcl-2. The blots were then developed using the ECL Plus kit.

Blots were then probed with primary antibodies and developed using the Enhanced Chemiluminescence-Plus detection system (Amersham Biosciences, Inc.). Primary antibodies included mouse anti-human c-Myc (Ab-1, OP10) and mouse anti-human p53 (Ab-1, OP03), both from Oncogene Research; mouse anti-human p21 (F-5, SC-6246) and mouse anti-human c-Myc (Ab-1, OP10) and mouse anti-human p53 (Ab-1, OP03), both from Santa Cruz Biotechnology; mouse anti-human c-Myc (C33, SC42), both from DAKO. Mouse anti-human p21, and mouse anti-human Bcl-2. The blots were then developed using the ECL Plus kit.

Chloromphenicol Acetyltransferase (CAT) Assay for c-myc, p53, and Control RSV Promoter Activity—Assays were performed as described previously (18). In brief, the construct/reporter plasmids pCBp53-CAT (17), 1.6Bgl myc-CAT (18), and Rous sarcoma virus (pRSV-CAT (19)) were transfected into cells by electroporation with a Bio-Rad Gene Pulser (250 V at a capacity setting of 960 microfarads). To normalize for different transfection efficiencies, a plasmid containing the β-galactosidase reporter gene driven by the actin promoter was cotransfected into the cells. After electroporation, cells were treated with the recombinant FN proteins V^+H^− and V^+H^+ or with control medium for different times. Cells were cultured with phosphate-buffered saline and lysed with lysis buffer (250 mM Tris-HCl, pH 7.5, 0.1% Triton X-100). CAT assays were performed as described previously (16). β-Galactosidase activity was measured by the Galacto-Light Plus chemiluminescent assay (Tropix, Bedford, MA) using a luminometer (Analytical Luminescence Laboratory, model 2010). CAT activity was normalized by β-galactosidase activity and compared for all treatments from triplicate experiments.

Transient Transfection of Cells—Cells that were 60—80% confluent in 96-well tissue culture plates were transiently transfected with 0.1 μg of DNA (as indicated in each figure legend) in 50 μl of serum-free medium or with vector control, using the LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer’s instructions. Transfection efficiency in these primary cells is ~30—35% as measured using a green fluorescent protein vector pEGFP (CLONTech). Transfected cells were then rinsed, fresh serum-free medium plus the FN protein (V^+H^−) was added to the test wells, and apoptosis was assessed. pCMV-jun (as described previously (20)) was used to construct cmv-c-myc, c-jun was removed, and human c-myc (2.5 kb) was inserted with HindIII.
FACS®-based Apoptosis Assay—The apoptotic population was assessed in primary human fibroblasts transfected with c-myc, p53, c-myc antisense, c-myc non-sense, p21, C-p21, or vector control and in p53−/− and wild-type mouse fibroblasts using a flow cytometric assay (11). Treated and control cells were resuspended in 1 ml of ice-cold phosphate-buffered saline containing 2% fetal calf serum, 3% enzyme-free phosphate-buffered saline-based cell dissociation buffer (Invitrogen), and 1 μg/ml propidium iodide (Sigma). Cells were kept on ice until 10–15 min before the addition of Hoechst 33342 stain. After equilibration to room temperature, 5 μg/ml Hoechst 33342 was added to the cell suspension. Detection of Hoechst 33342 staining was done after 6 min using a dual laser Triple Vantage S.E. cell sorter (Becton Dickinson, San Jose, CA).

RESULTS

p53 and c-Myc Protein and RNA Are Down-regulated in V+H− Fragment-mediated Apoptosis—In primary human fibroblasts (11), we initially observed that a recombinant fragment of FN containing the alternatively spliced V region and a mutation in the high-affinity heparin-binding domain (2) induced apoptosis in these cells (Fig. 1, a–c). This apoptotic pathway is proteoglycan- and caspase-mediated and is associated with changes in pp125FAK phosphorylation (11). On examining p53 and c-Myc function, we found surprising results, namely that p53 and c-Myc protein (Fig. 1d) and RNA levels

Fig. 2. Correspondence between down-regulation of c-Myc and p53 proteins and RNA levels by the V+H− FN fragment. a, RNA levels for c-Myc and p53 were analyzed by Northern blots at 3 and 7 h using standard methods. Blots were probed with denatured radiolabeled cDNA probes for human c-myc or human p53. Blots were normalized for differences in RNA loading and/or transfer to the membranes by stripping and rehybridizing with a 1.3-kb GAPDH cDNA probe. Results are expressed as the ratio of the c-Myc or p53 mRNA to the GAPDH mRNA signal. b, CAT reporter assays were used to assess the activity of the p53, c-myc, and control RSV promoters. Representative experiments are presented for both Northern blots and promoter assays. Values represent means and S.D. for three experiments. Data were evaluated statistically using one-way analysis-of-variance with the Newman-Keuls test for significance by GraphPad InStat software. *, p < 0.05, and **, p < 0.01, versus control.
FIG. 3. Rescue of FN fragment-mediated apoptosis by ectopic expression of c-Myc and p53. a, primary human fibroblasts were transfected with CMV-p53 (34), human CMV-c-myc, or vector controls using the LipofectAMINE Plus kit. Then cells were treated with the V^−H^− FN fragment for 3 h and photographed. b, primary fibroblasts transfected as described in a with p53 and c-myc were rescued from apoptosis, whereas control-transfected cells were not. Apoptosis was assessed by a flow cytometric assay. PI, propidium iodide; Hoechst, Hoechst 33,342. In each plot, the events represented in red (upper gated area) correspond to PI/Hoechst ratios characteristic of an apoptotic population, whereas the events represented in green (lower gated area) correspond to nonapoptotic cells (14). c, the fold change in apoptosis following transfection with c-myc, p53, and vector control were computed relative to untreated comparable samples for an average of three experiments and illustrated graphically. Values represent means and S.D. Data were evaluated statistically as described in the legend for Fig. 2. **, p < 0.01 versus vector-transfected cells. d, Western immunoblots for p53 and c-Myc of cells in panel a.
FIG. 4. Triggering a faster rate of apoptosis by p53-deficient conditions. *a*, primary p53+/+ and mutant p53−/− mouse fibroblasts were incubated in medium alone (CTL) or with the V−H+ fragment and then photographed after 30 min. *b*, Western immunoblots for p53 and c-Myc of cells in panel a except cell lysate samples were collected at 1 and 3 h. Membranes were developed with the ECL Plus kit. *c*, p53−/− mouse fibroblasts underwent a faster rate of apoptosis compared with p53+/+ mouse fibroblasts. Apoptosis was assessed by a flow cytometric assay as described in Fig. 3. *d*, the fold change in apoptosis for the p53−/− and p53+/+ mouse fibroblasts were computed relative to untreated comparable samples, for an average of three experiments and illustrated graphically. Values represent means and S.D. Data were evaluated statistically as described in the legend for Fig. 2. **, p < 0.01 versus p53−/− conditions at the comparable time point.
FIG. 5. Triggering a faster rate of apoptosis by c-myc antisense conditions. a, c-myc-, antisense-, and non-sense-transfected cells were incubated with the V/H fragment and then photographed after 1 h. b, Western immunoblot illustrates the effect of the oligonucleotides on c-Myc expression of cells in panel a except cell lysates were collected at 1 and 3 h. Control (CTL) cells were transfected with Oligofectin without oligonucleotides. Membranes were developed with the ECL Plus kit. NS, non-sense; AS, antisense. c, antisense-transfected cells underwent a faster rate of apoptosis compared with non-sense-transfected and control-transfected cells. Apoptosis was assessed by a flow cytometric assay as described in Fig. 3. d, the fold change in apoptosis for antisense-, non-sense-, and control-transfected cells were computed relative to untreated comparable samples for an average of three experiments and illustrated graphically. Values represent means and S.D. Data were evaluated statistically as described in the legend for Fig. 2. **, p < 0.01 versus antisense-transfected cells at 3 h.
FIG. 6. Rescue of the apoptotic phenotype by ectopic expression of full-length p21 but not the C-terminal portion of p21 (C-p21). 
a, human fibroblasts were untransfected, p21-transfected, or C-p21-transfected using the LipofectAMINE Plus kit. Cells were then treated with the V+H-FN fragment for 3 h and photographed. b, the p21-transfected cells showed a lower rate of apoptosis compared with the untransfected control and C-p21-transfected cells. Apoptosis was assessed by a flow cytometric assay as described in the legend for Fig. 3. c, the fold changes in apoptosis for p21- and C-p21-transfected cells, and untransfected cells were computed relative to untreated comparable samples for an average of three experiments and illustrated graphically. Values represent means and S.D. Data were evaluated statistically as described in the legend for Fig. 2. **, $p < 0.01$ versus C-p21-transfected cells and untransfected cells. d, Western immunoblots of p21 and C-p21 of the cells in panel a.
(Fig. 2a) were down-regulated as part of this apoptotic mechanism. However, levels of Bcl-2, an antiapoptotic protein, remained unchanged under the same conditions (Fig. 1d), illustrating the specificity of the p53 and c-Myc response. In addition, when cells were treated with the control FN protein V’H- or control medium, no apoptosis was triggered, and p53 and c-Myc levels remained unchanged (Figs. 1 and 2). Time course experiments demonstrated that the drop in c-Myc levels preceded that of p53, because the c-Myc signal began its downward trend at 30 min, whereas the p53 signal started to decline at 45 min (data not shown).

The Decline in p53 and c-Myc Is Transcriptionally Regulated—To determine whether the decrease in p53 and c-Myc levels was a transcriptionally regulated event, primary human fibroblasts were first transfected with a p53 promoter/reporter-CAT construct, a c-myc promoter/reporter-CAT construct, or a control RSV-CAT promoter/reporter construct and then treated with the apoptosis-inducing FN fragment, V’H-. After transfection with the p53 or c-myc promoters, promoter activity declined (Fig. 2b) mirroring the decline in the corresponding protein and RNA species and illustrating the transcriptional down-regulation of p53 and c-Myc in this pathway. However, RSV promoter activity was not down-regulated under the same conditions, further illustrating the specificity of the p53 and c-Myc response.

Transfection with p53 or c-myc Rescues the Apoptotic Phenotype—Suspecting that the decrease in p53 and c-Myc was driving the FN fragment-mediated apoptotic pathway, we transfected primary human fibroblasts with p53, c-myc, or vector controls to determine whether ectopic expression of p53 or c-Myc could rescue the apoptotic phenotype. Indeed, transfection with either p53 or c-myc, but not the control vector, rescued cells from FN fragment-mediated apoptosis (Fig. 3, a–c). Cell shape changes for the c-myc- and p53-transfected cells demonstrated a more well spread phenotype, characteristic of live cells, whereas the vector-transfected cells demonstrated cell membrane blebbing and a rounded phenotype associated with apoptosis (Fig. 3a). The c-myc-transfected cell population showed a 10.8% base-line level of apoptosis, which increased to 14% upon treatment with the V’H- protein. The p53-transfected cell population showed a 9.5% base-line level of apoptosis, which increased to 10.8% upon treatment with the V’H- protein. The vector-transfected control cell population showed a 5.1% base-line level of apoptosis, which increased to 15% upon treatment with the V’H- protein. Thus, the c-myc- and p53-transfected cells showed a 1.3-fold (14/10.8%) and 1.1-fold (10.8/9.5%) increase in apoptosis, respectively, compared with the 2.9-fold (15/5.1%) increase in apoptosis in the vector-transfected cells (Fig. 3b). This is approximately a 2.5-fold difference. Although, the rescue from apoptosis was not complete with c-myc and p53 transfection (and with other treatments; Fig. 4-6), this likely represents an incompletely transfected cell population; transfection efficiency is ~30–35% in these primary cells (data not shown). The fold changes in apoptosis following transfection with c-myc, p53, and vector control were computed for an average of 3 experiments and graphically illustrated (Fig. 3c). Confirmatory Western immunoblots indicate the higher expression levels for p53 and c-Myc achieved in the respective transfected cells (Fig. 3d). The relatively unchanged levels of p53 protein in the c-myc-transfected cells suggest that c-Myc may not be directly upstream of p53 or in its direct regulatory pathway.

p53-deficient Cells Undergo a Faster Rate of Apoptosis—To determine whether it is the down-regulation of the p53 signal that is required to mediate this mechanism, p53-deficient mouse fibroblasts (p53-/-) and wild-type (p53+/+) controls were treated with the apoptosis-inducing FN fragment and examined for their rate of apoptosis. The p53-deficient cells underwent a faster rate of apoptosis than their p53+/+ counterparts (Fig. 4, a, c, and d). Cell shape changes for the primary mouse fibroblasts demonstrated a more well spread phenotype, characteristic of live cells, whereas the p53+-/- fibroblasts demonstrated cell membrane blebbing and a rounded phenotype, associated with apoptosis (Fig. 4a). The wild-type fibroblasts showed a 3.3% base-line level of apoptosis, which increased to 5% at 1.5 h and 5.8% at 3 h upon treatment with the V’H- protein. The p53-/- fibroblasts showed a 3.6% base-line level of apoptosis, which increased to 12% at 1.5 h and 14.2% at 3 h upon treatment with the V’H-, a 3.3- and 3.9-fold increase in apoptosis at 1.5 h and 3 h, respectively, compared with the 1.5- and 1.8-fold increase in apoptosis in the wild-type fibroblasts at the same respective time points (Fig. 4c). This is approximately a 2-fold difference. The fold changes in apoptosis for the p53-/- and wild-type fibroblasts were computed for an average of three experiments and illustrated graphically (Fig. 4d). Western immunoblots confirmed the absence of p53 in the p53-/- cells and the decrease in p53 and c-Myc proteins in the normal mouse fibroblasts after treatment with the V’H- fragment (Fig. 4b). In p53-/- cells, c-Myc protein levels did not change appreciably or were only slightly decreased in response to the V’H- treatment, suggesting that although c-Myc may not be in a direct regulatory pathway with p53, it may be influenced by p53 status in this apoptotic mechanism.

c-myc Antisense Conditions Trigger a Faster Rate of Apoptosis—The requirement for down-regulation of c-Myc in this apoptotic pathway was also investigated by using antisense strategies. In this case, c-myc antisense-treated human fibroblasts underwent a more rapid rate of apoptosis than control nonsense oligonucleotide-treated or control-transfected cells upon addition of the V’H- fragment (Fig. 5, a, c, and d), further confirming that it is in part the depressed levels of c-Myc that mediate this mechanism. Cell shape changes for the non-sense-treated cells demonstrated a more well spread phenotype, characteristic of live cells, whereas the c-myc antisense-treated cells demonstrated cell membrane blebbing and a rounded phenotype (Fig. 5a). The antisense-treated cells showed a 1.4% base-line level of apoptosis, which increased to 3.9% at 1 h and 7.9% at 3 h upon treatment with the V’H- protein. The non-sense-treated cells showed a 2% base-line level of apoptosis, which increased to 2.8% at 1 h and 5.9% at 3 h upon treatment with the V’H- protein. The control-transfected cells showed a 3.3% base-line level of apoptosis, which increased to 5.4% at 1 h and 8% at 3 h upon treatment with the V’H- protein. Thus, the antisense-treated cells showed a 2.8- and 5.6-fold increase in apoptosis at 1 and 3 h, respectively, compared with the 1.4- and 2.95-fold increase in apoptosis in the non-sense-treated cells at the same respective time points. Similarly, the transfected controls showed a 1.6- and 2.4-fold increase in apoptosis. Therefore, both of the controls showed an ~2-fold lower level of apoptosis than the antisense-treated cells (Fig. 5c). The fold changes in apoptosis for antisense-, non-sense-, and control-transfected cells were computed for an average of three experiments and illustrated graphically (Fig. 5d). Fig. 5b illustrates the effect of the oligonucleotides on c-Myc expression. Taken together, these data suggest that down-regulation of either p53 or c-Myc may be sufficient for induction of apoptosis by the FN fragment because loss of either p53 or c-Myc function was sufficient to induce a faster rate of apoptosis.

p21 Levels Are Depressed and Transfection with p21 Rescues the Apoptotic Phenotype—Consistent with the changes in p53, levels of a downstream signal, cyclin-dependent kinase p21, were depressed in tandem (Fig. 1d). Ectopic expression of full-
length p21, but not its C-terminal form (C-p21) (22), rescued the apoptotic phenotype induced by the V' H' fragment (Fig. 6, a–c). The p21-transfected cells demonstrated the more well spread phenotype characteristic of live cells, whereas the C-p21-transfected and untransfected control cells demonstrated a rounded and cell membrane blebbing phenotype (Fig. 6a). The p21-transfected cell population showed a 2.4% baseline level of apoptosis, which increased to 4.8% upon treatment with the V' H' protein. The C-p21-transfected cell population showed a 2.2% baseline level of apoptosis, which increased to 9.7% upon treatment with the V' H' protein. The untransfected cell population showed a 2.7% base-line level of apoptosis, which increased to 11.3% upon treatment with the V' H' protein. Thus, the p21-transfected cells showed only a 2-fold increase in apoptosis, compared with the 4.2- and 4.4-fold increase in the untransfected control and C-p21-transfected cells, respectively. Therefore, the p21-transfected cells showed approximately a 2-fold lower level of apoptosis than the controls (Fig. 6b). The fold changes in apoptosis for p21- and C-p21-transfected cells, and untransfected cells were computed for an average of three experiments and illustrated graphically (Fig. 6c). Western immunoblot results confirmed the increased expression levels of p21 in the p21-transfected cells and the more gradual decline of p21 protein in these same cells upon treatment with the FN fragment (Fig. 6d). The decline in p21 was not surprising given that p53 acts as a transcriptional activator of p21. In addition, p53 protein levels remained unchanged after p21 transfection, again suggesting that p53 lies upstream of p21 in this pathway.

**DISCUSSION**

Although a great deal is known about how the p53 protein interacts with other proteins to control transcription, little is known about the factors that control p53 transcription itself. However, our data shed light on how p53 transcription can be regulated by ECM-generated signals and specifically by the heparin-binding domain and alternatively spliced V region of FN.

Our combined data suggest an alternative regulatory pathway for p53, c-Myc, and p21 as part of an apoptotic pathway initiated by the interactions of an altered ECM ligand with cell surface receptors. Proteoglycan and integrin receptors (11) then trigger an apoptotic signaling pathway that leads to decreases in pp125 

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