RhoA Function in Lamellae Formation and Migration Is Regulated by the α6β4 Integrin and cAMP Metabolism

Kathleen L. O’Connor, Bao-Kim Nguyen, and Arthur M. Mercurio
Division of Cancer Biology and Angiogenesis, Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215

Abstract. Clone A colon carcinoma cells develop fan-shaped lamellae and exhibit random migration when plated on laminin, processes that depend on the ligation of the α6β4 integrin. Here, we report that expression of a dominant negative RhoA (N19RhoA) in clone A cells inhibited α6β4-dependent membrane ruffling, lamellae formation, and migration. In contrast, expression of a dominant negative Rac (N17Rac1) had no effect on these processes. Using the Rhotekin binding assay to assess RhoA activation, we observed that engagement of α6β4 by either antibody-mediated clustering or laminin attachment resulted in a two- to threefold increase in RhoA activation, compared with cells maintained in suspension or plated on collagen. Antibody-mediated clustering of β1 integrins, however, actually suppressed RhoA activation. The α6β4-mediated interaction of clone A cells with laminin promoted the translocation of RhoA from the cytosol to membrane ruffles at the edges of lamellae and promoted its colocalization with β1 integrins, as assessed by immunofluorescence microscopy. In addition, RhoA translocation was blocked by inhibiting phosphodiesterase activity and enhanced by inhibiting the activity of cAMP-dependent protein kinase. Together, these results establish a specific integrin-mediated pathway of RhoA activation that is regulated by cAMP and that functions in lamellae formation and migration.

Key words: carcinoma • protein kinase A • G-protein • phosphodiesterase • cytoskeleton

Introduction

The organization and remodeling of the actin cytoskeleton are controlled by the Rho family of small GTPases, which includes Rho, Rac, and cdc42. These proteins have been implicated in the formation of stress fibers, lamellipodia, and filopodia, respectively (reviewed in Hall, 1998). Although much of our knowledge on their function has been obtained from studies with fibroblasts, other activities for these Rho GTPases have been observed recently in cells of epithelial origin. For example, Rac and cdc42 are required to maintain apical–basal polarity in epithelia (Jou and Nelson, 1998). Rho, interestingly, has been implicated in membrane ruffling in epithelial cells (Nishiyama et al., 1994; Fukata et al., 1999), a process attributed to Rac in fibroblasts (Hall, 1998). These findings are of particular interest with respect to our understanding of epithelial cell migration. The migration and invasion of epithelial-derived carcinoma cells are important phenomena that require the involvement of Rho GTPases (Keely et al., 1997; Shaw et al., 1997; Yoshioka et al., 1998; Itoh et al., 1999). For these reasons, it is essential to define the factors that regulate the function of Rho GTPases in carcinoma cells and to characterize the mechanisms by which they contribute to the dynamics of migration. For example, although cell adhesion has been reported to activate RhoA (Barry et al., 1997; Ren et al., 1999), little is known about the involvement of specific integrins in adhesion-dependent RhoA activation or in the regulation of RhoA-dependent functions.

Recent studies by our group have highlighted a pivotal role for the integrin α6β4 in the migration and invasion of carcinoma cells, as well as in epithelial wound healing (Lotz et al., 1997; Rabinovitz and Mercurio, 1997; Shaw et al., 1997; O’Connor et al., 1998; Rabinovitz et al., 1999). Although it is well established that α6β4 functions in the formation and stabilization of hemidesmosomes (Borradori and Sonnenberg, 1996; Green and Jones, 1996), our findings revealed a novel role for this integrin in the formation of actin-rich cell protrusions at the leading edges of carcinoma cells and in the migration of these cells...
(Rabinovitz and Mecurio, 1997; Rabinovitz et al., 1999). Moreover, we demonstrated the importance of CaMP metabolism in these events (O’Connor et al., 1998). Given the recent interest in the participation of RhoA in migration, we examined the hypothesis that RhoA is essential for the formation of actin-rich cell protrusions, the migration of carcinoma cells and, more importantly, that the activity of RhoA is regulated by the α6β4 integrin. In addition, we assessed the involvement of CaMP metabolism in these events.

Materials and Methods

Cells and Antibodies

Clone A cells, originally isolated from a poorly differentiated colon adenocarcinoma (Deexter et al., 1979), were used in all experiments. For each experiment, adherent cells were harvested by trypsinization, rinsed three times with RPMI medium containing 250 μg/ml heat-inactivated BSA (RPMI/BSA), and resuspended in RPMI/BSA. Where indicated, cells were treated with 1 mM isobutylmethylxanthine (IBMX) or 15 mM H-89 (Calbiochem-Novabiochem, Int.) for 15 min before use. The following antibodies were used in this study: MC13, mouse anti-β1 integrin mAb (obtained from Steve Akiyama, National Institutes of Health, Research Triangle Park, NC); K20, mouse anti-β1 integrin mAb (immunotech); 439-9B, rat anti-β4 integrin mAb (obtained from R. Ita Falcioni, Regina Elena Cancer Institute, Rome, Italy); mouse anti-HA mAb (Roche Biochemicals); rabbit anti-RhoA polyclonal antibody (Santa Cruz Biotechnology); and anti-Rac1 (Transduction Laboratories).

To obtain expression of N19RhoA and N17Rac1, adherent cells were harvested using trypsin, rinsed with PBS, and suspended in electroporation buffer (20 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4·7H2O, 0.5 mM glucose). Cells were cotransfected with 1 mg of either pCMVβ-gal or pGFP (green fluorescent protein) and 4 μg of either control vector or vector containing HA-tagged N19RhoA (provided from Alex Toker, Beth Israel Deaconess Medical Center, Boston, MA) or GST-tagged N17Rac1 (obtained from Margaret Chou, University of Pennsylvania) by electroporation at 250V and 500 μFd. Subsequently, cells were plated in complete growth medium containing 0.05% sodium butyrate and used for experiments 48 h after the initial transfection. Expression of the recombinant proteins was confirmed by concentrating extracts of transfected cells with an HA-specific mAb or glutathione-agarose, and used for experiments 48 h after the initial transfection. Expression of the recombinant proteins was confirmed by concentrating extracts of transfected cells with an HA-specific mAb or glutathione-agarose, and used for experiments 48 h after the initial transfection.

Microscopic Analyses

Glass coverslips were coated overnight at 4°C with collagen I (50 μg/ml; Collagen Corp.) or laminin-1 purified from EHS tumor (20 μg/ml; provided by Hinda K Leinman, NIDR, Bethesda, MD) and then blocked with BSA (0.2% in RPMI). Cells were plated on these coverslips for 30–40 min, rinsed with PBS, fixed, and then permeabilized as described previously (O’Connor et al., 1998). For immunofluorescence, cells were incubated with 1 μg/ml of K20 (anti-β1) and anti-RhoA antibody diluted in block solution (3% BSA/1% normal donkey serum in PBS) for 30 min, rinsed four times with PBS, and then incubated for 30 min with a 1:400 dilution of anti-mouse IgG Cy2- and anti-rabbit IgG Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Images of cells were captured digitally, analyzed, and lamellar area quantified as described previously (Rabinovitz and Mecurio, 1997; O’Connor et al., 1998).

Migration Assays

The lower compartments of Transwell chambers (6.5-mm diam, 8-μm pore size; Costar) were coated for 30 min with 15 μg/ml laminin-1 diluted in RPMI medium. RPMI/BSA was added to the lower chamber and cells (1 × 10⁴) suspended in RPMI/BSA were added to the upper chamber. After incubating for 5 h at 37°C, cells were removed from the upper chamber with a cotton swab and cells that had migrated to the lower surface of the membrane were fixed, stained with crystal violet or for β-galactosidase (β-gal), and quantified as described previously (Shaw et al., 1997).

RhoA Activity

RhoA activity was assessed using the Rho-binding domain of Rhotekin as described (Ren et al., 1999). In brief, cells (3 × 10⁴) were plated onto 60-mm dishes coated with LN-1 (20 μg/ml) or collagen I (50 μg/ml) for 30 min and extracted with RIPA buffer (50 mM Tris, pH 7.2, 500 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1% SDS, 10 mM MgCl₂, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 4 μg/ml aprotinin, and 2 mM PMSF). Alternatively, cells were incubated with 8 μg of anti-ß1 mAb mc13 or anti-ß4 rat mAb 439-9B for 30 min, rinsed, plated on 60-mm dishes coated with 50 μg of either anti-mouse or anti-rat IgG, respectively, for 30 min, and then extracted. A further centrifugation at 14,000 g for 3 min, the extracts were incubated for 45 min at 4°C with glutathione beads (Pharmacia Biotech) coupled with bacterial expressed GST-RBD (Rho-binding domain of Rhotekin) fusion protein (provided by Martin Schwartz, Scripps Research Institute, La Jolla, CA), and then washed three times with Tris buffer, pH 7.2, containing 1% Triton X-100, 150 mM NaCl, and 10 mM M gC₄. The RhoA content in these samples was determined by immunoblotting samples using rabbit anti-RhoA antibody.

Results

Clone A colon carcinoma cells develop fan-shaped lamellae and exhibit random migration when plated on laminin-1, processes that are dependent on both the α6β4 and β1 integrins. In contrast, the β1 integrin-mediated adhesion and spreading of these cells on collagen I does not induce significant lamellae formation or migration (Rabinovitz and Mecurio, 1997; Shaw et al., 1997). To examine the hypothesis that RhoA functions in α6β4-dependent lamellae formation, clone A cells were cotransfected with a GFP construct and either a dominant negative RhoA (N19RhoA) or a control vector. Subsequently, the cells were plated onto laminin-1 and examined by phase-contrast microscopy. Clone A cells that expressed the control vector developed large lamellae with ruffled edges (Fig. 1 A). In contrast, cells that expressed N19RhoA developed only a few small, fragmented lamellae that were devoid of membrane ruffles (Fig. 1 B). Quantitative analysis of these images revealed that expression of N19RhoA reduced lamellar area by 80% in comparison to cells that expressed the control vector (Fig. 1 D). Interestingly, expression of a GST-tagged, dominant negative Rac1 (N17Rac1) did not inhibit either lamellae formation or membrane ruffling in clone A cells (Fig. 1 C and D), although this construct has been shown to inhibit p70 S6 kinase (Chou and Blenis, 1996) and invasion (Shaw et al., 1997).

Expression of N19RhoA inhibited the migration of clone A cells on laminin-1 by 70% (Fig. 2 A). In contrast, expression of N17Rac1 did not inhibit the migration of clone A cells (Fig. 2 A), although it did inhibit the migration of 3T3 cells by 85% (data not shown). Importantly, expression of N19RhoA had only a modest effect on cell spreading because cells expressing N19RhoA plated on collagen-1 spread to ~80% of the surface area occupied by control cells (Fig. 1 E). Expression of N19RhoA and N17Rac1 in clone A cells was confirmed by immunoblotting (Fig. 1 F and G).

Our observation that RhoA functions in lamellae for-
The involvement of cAMP metabolism in migration, lamellae formation, and α6β4-mediated activation of RhoA was investigated using both IBMX, a phosphodiesterase (PDE) inhibitor, and H-89, a cAMP-dependent protein kinase (PKA) inhibitor. IBMX treatment, which prevents cAMP breakdown, inhibited the migration of clone A cells on laminin-1 almost completely (Fig. 2 B). In contrast, inhibition of PKA with H-89 increased the rate of migration on laminin-1 as described in Materials and Methods. Migration was scored as the relative number of β-gal staining cells migrated compared with the vector only control. Transfection rates were comparable. B, Clone A cells were left untreated or treated with 1 mM IBMX or 15 μM H-89 for 15 minutes and then assayed for laminin-1 mediated migration as described in Materials and Methods. Migration rates were reported as the number of cells migrated per mm². Bars represent mean ± SD from triplicate determinations.

Figure 2. Effects of dominant negative RhoA (N19RhoA) and cAMP metabolism on laminin-1 stimulated migration. A, Clone A cells that had been cotransfected with a β-gal cDNA and either N19RhoA, N17Rac1, or control vector were assayed for migration on laminin-1 as described in Materials and Methods. Migration was scored as the relative number of β-gal staining cells migrated compared with the vector only control. Transfection rates were comparable. B, Clone A cells were left untreated or treated with 1 mM IBMX or 15 μM H-89 for 15 minutes and then assayed for laminin-1 mediated migration as described in Materials and Methods. Migration rates were reported as the number of cells migrated per mm². Bars represent mean ± SD from triplicate determinations.
RhoA, we used IBMX in the RBD assay. As shown in Fig. 3 A, pretreatment of clone A cells with IBMX before plating on laminin-1 reduced the level of RhoA activation to that observed in cells plated on collagen. Importantly, IBMX did not inhibit either cell adhesion or spreading (Fig. 4, C and E). Similar results were obtained with integrin clustering (data not shown). These observations implicate cAMP metabolism in the α6β4-mediated activation of RhoA.

The data reported here raise the possibility that α6β4 influences RhoA localization because activation of RhoA is thought to involve its translocation to membranes (Bokoch et al., 1994). To address this issue, clone A cells plated on either laminin-1 or collagen I were immunostained with a RhoA-specific antibody, as well as a β1-integrin-specific antibody to mark membranes. In cells plated on collagen I, RhoA immunostaining was confined largely to the cytosol and it was distinct from the β1-integrin staining of the plasma membrane (Fig. 4 A). In contrast, the α6β4-dependent interaction of clone A cells with laminin-1 resulted in the translocation of RhoA to membrane ruffles at the edges of lamellae where it colocalized with β1 integrin staining (Fig. 4 B). However, RhoA did not colocalize with β1 integrins on the plasma membrane along the cell body (Fig. 4 B). To assess the influence of cAMP metabolism on RhoA localization, clone A cells were pretreated with either IBMX or H-89 before plating on laminin-1. Inhibition of PDE activity with IBMX dramatically inhibited membrane ruffling and abolished RhoA localization in the few ruffles that persisted after IBMX treatment (Fig. 4, C and E). Conversely, inhibition of cAMP-dependent PKA with H-89 resulted in an apparent increase in membrane ruffling and RhoA localization in membrane ruffles (Fig. 4, D and F).

Discussion

Recently, we established that the α6β4 integrin stimulates the migration of carcinoma cells and enhances the formation of actin-rich protrusions, including lamellae and membrane ruffles (Shaw et al., 1997; Rabinovitz and Mercurio, 1997; O’Connor et al., 1998; Rabinovitz et al., 1999). In this study, we advance our understanding of the mechanism by which α6β4 functions in these dynamic processes by demonstrating that ligation of α6β4 with either antibody or laminin-1 results in the activation of RhoA and its translocation from the cytosol to membrane ruffles at the leading edges of migrating carcinoma cells. Importantly, we also provide evidence that the α6β4-mediated activation of RhoA is necessary for lamellae formation, membrane ruffling, and migration. Furthermore, we establish that these events are regulated by cAMP metabolism and that they can occur independently of Rac1 involvement.

Our findings strengthen the evidence that integrins can participate in the activation of RhoA. Much of the evidence supporting integrin activation of RhoA has been based largely on the observation that integrin activation leads to the Rho-dependent formation of stress fibers and focal adhesions (Ren et al., 1999; Schoenwaelder and Burridge, 1999). Recently, the development of a biochemical assay for RhoA activation using the ability of GTP-bound RhoA to associate with the Rho-binding domain of Rhotekin has enabled a more rigorous and sensitive assessment of the mechanism of RhoA activation (Ren et al., 1999). Using this assay, cell attachment to fibronectin was shown to activate RhoA and that the level of activation was augmented by serum or lysophosphatidic acid (LPA). In our study, we extend this observation by providing evidence that a specific integrin, α6β4, can activate RhoA, as assessed by both Rhotekin binding and translocation to membrane ruffles. An interesting and unexpected finding obtained in our study is that the α6β4 integrin is a more effective activator of RhoA than β1 integrins in clone A cells. In fact, antibody-mediated ligation of β1 integrins actually suppressed RhoA activation. Because we used carcinoma cells in our study, the potent activation of RhoA we observed in response to α6β4 ligation could have resulted from a cooperation of α6β4 with a secreted growth factor or activated oncogene. However, if cooperative signaling between integrins and such factors occurs in these cells, it is specific for α6β4 because clustering of β1 integrins did not activate RhoA.

Our findings implicate an important role for RhoA in the formation of membrane ruffles and lamellae. Specifically, the expression of N19RhoA in clone A cells attached to laminin resulted in the appearance of frag-
Rho kinase pathway is an important component of carcinoma progression. It is also possible that the α6β4-mediated activation of RhoA contributes to migration and invasion by activating the adhesive functions of other integrins. RhoA can activate integrin-mediated adhesion in leukocytes (Laudanna et al., 1997) and is believed to participate in adhesion in other cell types (Nobes and Hall, 1999). Our observation that RhoA and β1 integrins colocalize in membrane ruffles in response to α6β4 ligation raises the possibility that RhoA influences the function of β1 integrins, which are essential for migration and invasion.

Interestingly, expression of the dominant negative Rho kinase pathway is an important component of carcinoma progression. It is also possible that the α6β4-mediated activation of RhoA contributes to migration and invasion by activating the adhesive functions of other integrins. RhoA can activate integrin-mediated adhesion in leukocytes (Laudanna et al., 1997) and is believed to participate in adhesion in other cell types (Nobes and Hall, 1999). Our observation that RhoA and β1 integrins colocalize in membrane ruffles in response to α6β4 ligation raises the possibility that RhoA influences the function of β1 integrins, which are essential for migration and invasion.

Interestingly, expression of the dominant negative
N 17R ac in clone A cells had no inhibitory effect on either membrane ruffling, lamellae formation, or migration. A though it is well established that Rac functions in lamelli podia formation in fibroblasts (Hall, 1998) and in the mig ration of several cell types (e.g., see Keely et al., 1997; Shaw et al., 1997; Nobes and Hall, 1999), recent studies have highlighted the complexity of Rac involvement in these dynamic processes. For example, Rac activation can also inhibit migration by promoting cadherin-mediated cell–cell adhesion (Hordijk et al., 1997; Sander et al., 1999) and by downregulating Rho activity (Sander et al., 1999). Nonetheless, Rac activation stimulates membrane ruffling under conditions in which it also promotes cell–cell adhesion (Sander et al., 1999). Clone A cells, therefore, may represent the first example of a cell type in which both membrane ruffling and migration are Rac-independent.

Our results highlight the importance of cAMP metabolism in the activation and localization of RhoA. Our finding that Rac inhibits RhoA activation and translocation to membrane ruffles is consistent with our previous report that linked the ability of α6β4 to promote carcinoma migration with its ability to alter cAMP metabolism (O’Connor et al., 1998). In addition, these results substantiate other studies that indicated an inhibitory effect of cAMP on RhoA activity (Lang et al., 1996; Laudanna et al., 1997; Dong et al., 1998). The basis for this inhibition may be the direct phosphorylation of RhoA by PKA (Lang et al., 1996). The basis for this inhibition may be the direct phosphorylation of RhoA by PKA (Lang et al., 1996). This work was supported by the United States Army Medical Research and Materiel Command Grants DAMD17-98-1-8033 (to K. L. O’Connor) and DAMD17-98-1-8034 (to H. M. Flinn, M. J. Humphries, D. R. Critchley, and A. J. Ridley). 1997. Requirement for Rho in integrin-mediated migration. Involvement of specific laminin isoforms and integrin receptors in wound closure of a transformed model epithelium. Am. J. Pathol. 147:1235–1244.

References

Bokoch, G. M., B. P. Bohl, and T.-H. Chuang. 1994. Guanine nucleotide exchange regulates membrane translocation of Rac/Rho GTP-binding proteins. J. Biol. Chem. 269:31674–31679.

Borrelli, L., and A. Sonnenberg. 1996. Hemidesmosomes: roles in adhesion, signalling and human disease. Curr. Opin. Cell Biol. 8:647–658.

Chou, M. M., and J. Blenis. 1996. The 70 kDa 56 kinase complexes with and is activated by the rho family G proteins cdc42 and Rac. Cell. 85:573–583.

Dexter, D. L., J. A. Barbosa, and P. Calabresi. 1999. N-, N-dimethylformamide-induced alteration of cell culture characteristics and loss of tumorigenicity in cultured human colon carcinoma cells. Cancer Res. 59:1020–1025.

Dong, J. M., T. Leung, E. M. Anser, and L. L. Lim. 1998. cAMP-induced morphological changes are counteracted by the activated RhoA small GTPases and the rho kinase ROCK-a. J. Biol. Chem. 273:22554–22562.

Fukata, Y., N. Oshiro, N. Inoshita, Y. Kawano, Y. Matsuoka, V. Bennett, Y. Matsuura, and K. K. Akiyoshi. 1999. Phosphorylation of adducin by rho kinase plays a crucial role in cell motility. J. Cell Biol. 145:347–361.

Green, K. J., and C. R. J. Jones. 1996. Desmosomes and hemidesmosomes: structure and function of molecular components. FASEB J. 10:871–881.

Hall, A. H. 1998. Rho GTPases and the actin cytoskeleton. Science. 279:509–514.

Hordijk, P. L., J. P. ten Klooster, R. Van Der Kammen, and J. G. ten Klooster. 1998. RhoA activation by increasing the activity of a cAMP-dependent protein kinase in KB cells. J. Cell Biol. 145:347–361.

Kaye, S. J., and X. Wang. 1998. Regulation of integrin function by PI3K. J. Biol. Chem. 273:22554–22562.

Lee, E. C., M. M. Lotz, G. D. Steele, and A. M. Mercurio. 1992. The integrin α6β4 is a laminin receptor. J. Cell Biol. 117:671–678.

Lotz, M. M., C. A. Korzelius, and A. M. Mercurio. 1997. Intestinal epithelial restitution. Involvement of specific laminin isoforms and integrin receptors in wound closure of a transformed model epithelium. Am. J. Pathol. 150:747–760.

Nishiyama, T., T. Sakai, K. Takai, M. Kato, H. Yaku, K. A. Araki, Y. Matsuura, and Y. Takai. 1994. Rac p21 is involved in insulin-induced membrane ruffling and rho p21 is involved in hepatocyte growth factor- and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced membrane ruffling in KB cells. Mol. Cell. Biol. 14:2427–2456.

Nobes, C. D., and A. Hall. 1999. Rho GTPases control polarity, protrusion, and adhesion during cell movement. J. Cell Biol. 144:1235–1244.

O’Connor, K. L., M. L. Shaw, and A. M. Mercurio. 1998. Release of cAMP by the α6β4 integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. J. Cell Biol. 142:1749–1760.

Rabinovitz, I., and A. M. Mercurio. 1997. The integrin α6β4 in carcinoma cell migration on laminin-1 by mediating the formation and stabilization of actin-containing motility structures. J. Cell Biol. 139:1873–1884.

Rabinovitz, I., A. Toker, and A. M. Mercurio. 1999. Protein kinase C-dependent mobilization of the α6β4 integrin from hemidesmosomes and its association with actin-rich cell protrusions drive the chemotactic migration of carcinoma cells. J. Cell Biol. 146:1147–1159.

Ren, K.-D., W. B. Kloss, and M. A. Schwartz. 1999. Regulation of small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMB (Eur. Mol. Biol. Organ.) J. 15:510–519.

Rabinovitz, I., A. Toker, and A. M. Mercurio. 1999. Protein kinase C-dependent mobilization of the α6β4 integrin from hemidesmosomes and its association with actin-rich cell protrusions drive the chemotactic migration of carcinoma cells. J. Cell Biol. 146:1147–1159.

Shaw, L. M., J. P. ten Klooster, R. Van Der Kammen, and J. G. ten Klooster. 1998. Regulation of small GTP-binding protein Rho by cell adhesion and the cytoskeleton. EMB (Eur. Mol. Biol. Organ.) J. 15:510–519.

Sander, E. C., M. M. Lotz, G. D. Steele, and A. M. Mercurio. 1992. The integrin α6β4 is a laminin receptor. J. Cell Biol. 117:671–678.

We thank Steve Akiyama, R. I. Falcon, H. H. Wang, A. Toker, and R. Bache for helpful discussions.

This work was supported by the United States Army Medical Research and Materiel Command Grants DAMD17-98-1-8033 (to K. L. O’Connor) and DAMD17-98-1-6199 (to A. M. Mercurio), as well as National Institutes of Health Grant CA 80789 (to A. M. Mercurio) and the Harvard Digestive Diseases Center.

Submitted: 18 October 1999
R revised: 17 December 1999
Accepted: 20 December 1999

The Journal of Cell Biology, Volume 148, 2000 258

8

10

12

14

16

18

20

22

24

26

28

30

32

34

36

38

40

42

44

46

48

50

52

54

56

58

60

62

64

66

68

70

72

74

76

78

80

82

84

86

88

90

92

94

96

98

100

102

104

106

108

110

112

114

116

118

120

122

124

126

128

130

132

134

136

138

140

142

144

146

148

150

152

154

156

158

160

162

164

166

168

170