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Expression of alpha V integrin is modulated by Epstein–Barr virus nuclear antigen 3C and the metastasis suppressor Nm23-H1 through interaction with the GATA-1 and Sp1 transcription factors

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Abstract

Epstein–Barr virus (EBV) is a lymphotrophic herpesvirus infecting most of the world's population. It is associated with a number of human lymphoid and epithelial tumors and lymphoproliferative diseases in immunocompromised patients. A subset of latent EBV antigens is required for immortalization of primary B-lymphocytes. The metastatic suppressor Nm23-H1 which is downregulated in human invasive breast carcinoma reduces the migration and metastatic activity of breast carcinoma cells when expressed from a heterologous promoter. Interestingly, the EBV nuclear antigen 3C (EBNA3C) reverses these activities of Nm23-H1. The alpha V integrins recognize a variety of ligands for signaling and are involved in cell migration and proliferation and also serve as major receptors for extracellular-matrix-mediated cell adhesion and migration. The goal of this study was to determine if Nm23-H1 and EBNA3C can modulate alpha V integrin expression and downstream activities. The results of our studies indicate that Nm23-H1 downregulates alpha V intregrin expression in a dose responsive manner. In contrast, EBNA3C can upregulate alpha V integrin expression. Furthermore, the study showed that the association of the Sp1 and GATA transcription factors with Nm23-H1 is required for modulation of the alpha V integrin activity. Thus, these results suggest a direct correlation between the alpha V integrin expression and the interaction of Nm23-H1 with EBNA3C.

Keywords: Alpha V integrin; Nm23-H1; EBNA3C; FAK; Metastasis

Introduction

Epstein–Barr virus (EBV) is a human gamma herpesvirus which predominantly targets B cells and epithelial cells and is associated with a number of human cancers including Burkitt's lymphoma, Nasopharyngeal carcinoma, Hodgkin's disease, AIDS-associated and transplant-associated immunoblastic lymphoma, and somewhat controversially invasive breast carcinoma (Brooks et al., 1997; Kieff, 1996; Rickinson and Kieff, 1996). EBV is also known to be the causative agent of infectious mononucleosis (Brooks et al., 1997; Kieff, 1996; Rickinson and Kieff, 1996). In vitro infection of B cells with EBV gives rise to lymphoblastoid cell lines (LCLs) which express a subset of 12 latent viral transcripts (Kieff, 1996; Rickinson and Kieff, 1996). These 12 transcripts encode for six nuclear antigens (EBNAs), three latent membrane proteins (LMPs), two early RNAs (EBERs) and the BARF transcripts (Kieff, 1996; Rickinson and Kieff, 1996). Of these six genes, EBNA1, LP, -2, -3A, -3C and LMP1 have been shown to be important or critical for growth transformation and immortalization of human primary B cells in vitro (Kaye et al., 1993; Tomkinson et al., 1993; Cohen et al., 1989).

The EBNA3 family contains a set of three genes tandemly arranged on the EBV genome. The three proteins primarily function as transcriptional regulators and contain similar structural motifs as well as a region of limited homology in the amino terminus (Kieff, 1996; Zhao et al., 1996). This domain contains the binding site for the cellular repressor RBP-Jκ also referred to as CSL (Robertson et al., 1996; Zhao et al.,

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1996). EBNA3C can prevent binding of RBP-Jκ to EBNA2 and can downregulate EBNA2-modified transactivation of the LMP1 promoter through association of RBP-Jκ (Lin et al., 2002; Marshall and Sample, 1995). Furthermore, EBNA3C can regulate Cp, the major latent promoter controlling EBNA expression, through RBP-Jκ and other co-repressors (Radkov et al., 1997). EBNA3C has also been shown to play a role in regulating the acetylation and co-activation activity of the p300/ProTα complex (Subramanian et al., 2002a,b) and function as a transcriptional repressor (Subramanian et al., 2002a,b; Zhao and Sample, 2000).

Reverse genetic studies of EBV demonstrated that addition of a stop codon after aa 365 in the EBNA3C open reading frame nullified the ability of EBV to growth transform primary B lymphocytes in vitro (Tomkinson et al., 1993). Cellular partners previously identified include the metastasis suppressor Nm23-H1 which bound to a short stretch of amino acids located between the glutamine- and proline-rich domains of EBNA3C (Subramanian and Robertson, 2002; Subramanian et al., 2001). This interaction between Nm23-H1 and EBNA3C has been shown to result in an increase in transcriptional activity when tethered to a responsive promoter (Subramanian et al., 2001). These results suggest that Nm23-H1 may possess transcriptional regulatory activities independent of a possible role in directly binding to DNA or through its interaction with EBNA3C. Interestingly, the presence of EBNA3C mediates the cellular translocalization of Nm23-H1 from a mostly cytoplasmic to a predominantly nuclear signal (Subramanian and Robertson, 2002) and reverses the antimigratory effects of Nm23-H1 (Subramanian and Robertson, 2002).

The Nm23 gene family is a closely related group of nucleoside dinucleotide phosphate kinases for which eight distinct genes are known in humans (Nm23-H1 to -H8) (Lacombe et al., 2000). The proteins are characterized by a wide variety of functions including transcriptional regulation, differentiation, proliferation and suppression of tumor metastasis (Lacombe et al., 2000). Importantly, changes in cellular levels of Nm23-H1 have been correlated with decreased metastasis in a number of cancers including breast, gastric and cervical cancers, and in vivo studies in nude mice have demonstrated a role in suppressing metastasis (Miyazaki et al., 1999; Tagashira et al., 1998; Leone et al., 1991, 1993). A significant amount of data implicates Nm23-H1 in the regulation of metastasis; however, the biochemical mechanism for this activity is still poorly understood.

Integrins are heterodimeric molecules composed of a noncovalently associated alpha and beta subunit exclusively recognizing ligands via an RGD amino acid sequence (Hynes, 1992). The ligands for alpha V integrins include vitronectin, bronectin, brinogen, von Willebrand factor and osteopontin (Stamenkovic, 2000; Zhang et al., 1993; Denhardt and Guo, 1993; Busk et al., 1992; Charo et al., 1990; Smith et al., 1990; Pytela et al., 1985). Interestingly, alpha V integrins are expressed on migratory cells such as metastatic melanomas and breast cancer cells and integrin expression correlates with metastasis (Filardo et al., 1995; Nip et al., 1990; Albelda et al., 1990). Additionally, αvβ3 is directly associated with matrix metalloproteinase 2 (MMP2) on melanoma cells which is thought to provide tumor cells with the coordinated matrix degradation and cellular motility, thus facilitating cellular invasion (Brooks et al., 1996).

The role of Nm23-H1 in regulating alpha V integrins has not been previously investigated, and there are no direct report indicating the relationship between Nm23-H1 and alpha V integrin except in some clinical findings. Importantly, increased expression of alpha V integrin correlates with EBV in infected B cells (Huang et al., 2000). Moreover, LMP1, LMP2 and EBNA2 expression has been shown to increase levels of alpha V integrin in LCLs (Huang et al., 2000). In this report, we show that the interaction of the essential EBV latent antigen EBNA3C with Nm23-H1 also leads to increased expression of alpha V integrin. Additionally, we show that Nm23-H1 in the absence of EBNA3C decreased the expression of alpha V integrin. These results provide in part an important clue towards understanding the mechanism by which EBNA3C reverses the effects of Nm23-H1 in regulating the proliferation and migration of infected cells.

Results

Nm23-H1 and EBNA3C can modulate the transcription of alpha V integrin promoter

Previous studies have shown that EBNA3C can reverse Nm23-H1-mediated suppression of cell migration (Subramanian et al., 2001). To further elucidate the mechanism by which this occurs, we wanted to determine whether either or both of these proteins could modulate the expression levels of alpha V integrin, a protein found to play a key role in cell migration and metastasis (Miyazaki et al., 1999; Tagashira et al., 1998; Leone et al., 1991, 1993). The effect of these proteins on the full-length alpha V integrin promoter inserted into the pGL2 basic vector was assessed by luciferase reporter assay. Expression vector pA3M-EBNA3C, pA3M-Nm23-H1 or both were transiently transfected into BJAB and DG-75 cells along with the pGL2, the reporter plasmid containing the alpha V integrin promoter. Total transfected DNA was balanced with empty vector, and transfection efficiency was monitored by the use of pEGFP and counting of transfected cells for GFP fluorescence. The results from this assay demonstrated that the Nm23-H1 downregulated the alpha V integrin promoter in a dose-dependent manner in both BJAB and DG75 B-cell lines (Figs. 1A and D). Interestingly, EBNA3C modestly activated the promoter in the context of these two B-cell lines from a heterologous promoter (Figs. 1B and E). However, when Nm23-H1 and EBNA3C were co-expressed, there was an observed synergistic activation of the promoter when compared to vector alone, thus nullifying the repressive effects of Nm23-H1 (Figs. 1C and F). This result indicates that EBNA3C and Nm23-H1 exhibit opposite effects in terms of regulation of the alpha V integrin promoter in an in vitro luciferase reporter assay but together they can enhance the activation of the promoter as seen in BJAB and DG75, respectively (Figs. 1C and F). To confirm the

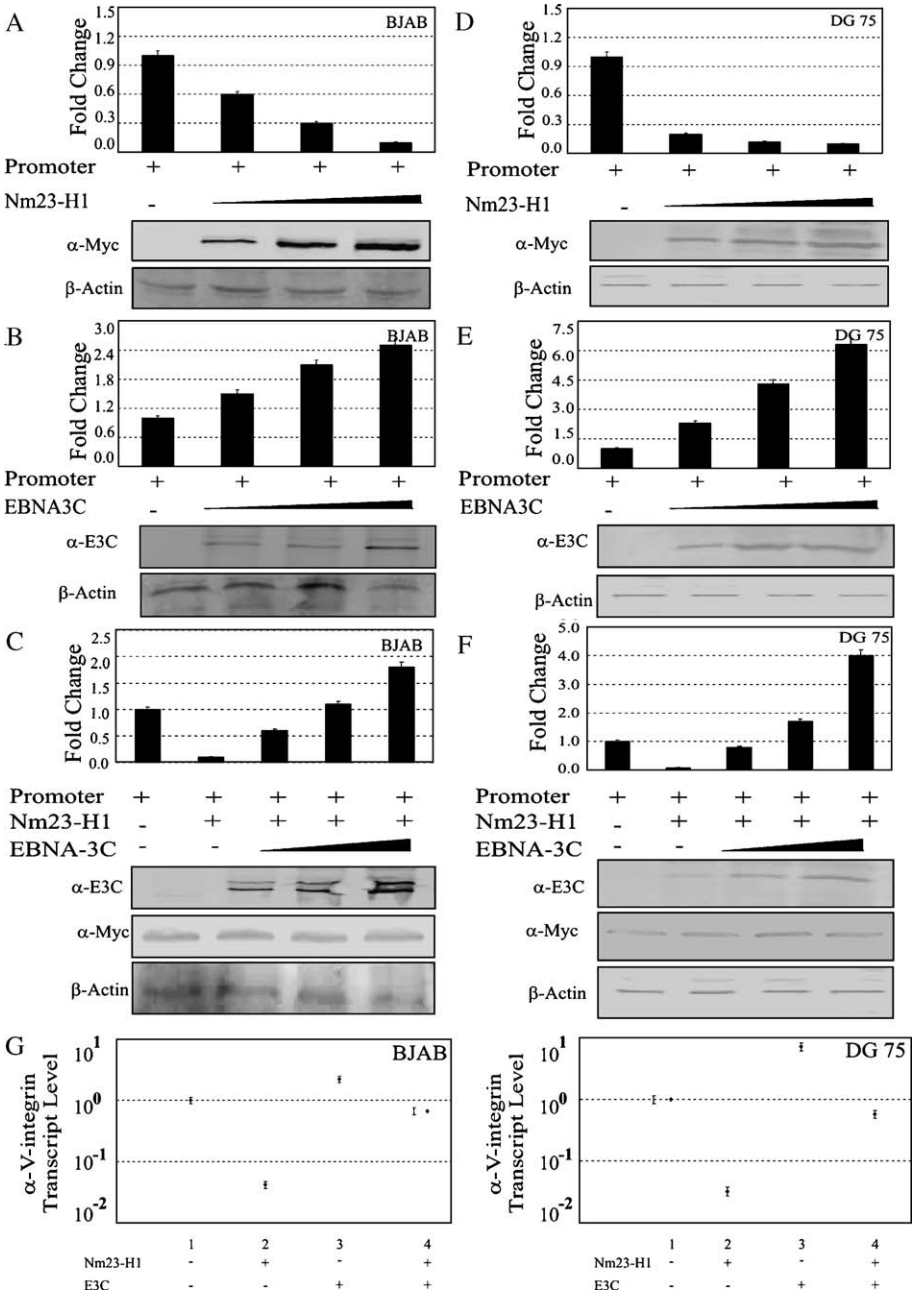


Fig. 1. Modulation of alpha V integrin promoter by Nm23-H1 and EBNA3C in BJAB and DG75 cells. (A) pGL2-alpha V integrin promoter (2.5 μg) was co-transfected with increasing amounts (2.5 μg, 5 μg, 7.5 μg) of pA3M-Nm23-H1, and 24 h post-transfected BJAB cells were harvested for luciferase assay. Increasing amounts of Nm23-H1 showed a dose-dependent decrease of promoter activity. (B) pGL2-alpha V integrin promoter (2.5 μg) was co-transfected with increasing amounts (2.5 μg, 5 μg, 7.5 μg) of pA3M-EBNA3C, and 24 h post-transfected BJAB cells were harvested for luciferase assay. Increasing amounts of EBNA3C showed a dose-dependent increase of promoter activity. (C) pGL2-alpha V integrin promoter (2.5 μg) was co-transfected with pA3M-Nm23-H1 (5 μg) and increasing amount (2.5 μg, 5 μg, 7.5 μg) of pA3M-EBNA3C. Twenty four hours post-transfected BJAB cells were harvested and lysed for luciferase assay. Increasing amount of EBNA3C showed an increase of promoter activity in the presence of constant amount of Nm23-H1. (D) pGL2-alpha V integrin promoter (2.5 μg) was co-transfected with increasing amounts (2.5 μg, 5 μg, 7.5 μg) of pA3M-Nm23-H1, and 24 h post-transfected DG75 cells were harvested for luciferase assay. Increasing amounts of Nm23-H1 showed a dose-dependent decrease of promoter activity. (E) pGL2-alpha V integrin promoter (2.5 μg) was co-transfected with increasing amounts (2.5 μg, 5 μg, 7.5 μg) of pA3M-EBNA3C, and 24 h post-transfected DG75 cells were harvested for luciferase assay. (G) pGL2-alpha V integrin promoter (2.5 μg) was co-transfected with pA3M-Nm23-H1 (5 μg) and increasing amount (2.5 μg, 5 μg, 7.5 μg) of pA3M-EBNA3C. 24 h post-transfected DG75 cells were harvested and lysed for luciferase assay. Fractions of lysates were used for Western blot with anti-EBNA3C to detect the EBNA3C and anti-myc for Nm23-H1 in all the data presented above. (G) Total RNA from 24 h post-transfected cells (vector only, Nm23-H1, EBNA3C and both Nm23-H1 and EBNA3C) DG75 and BJAB was used to make cDNA. The specific primers for alpha V. The cDNA was amplified using SYBR green real-time master mix. Data show that in Nm23-H1 overexpressing cells the level of alpha V integrin reduced to greater than 50-fold over vector alone and in EBNA3C overexpressed cells, there were a small increase in activity. In EBNA3C and Nm23-H1 co-expressing cells, transcript level was close to the vector alone. Means and standard deviations were derived from three independent experiments in all the above presented data.

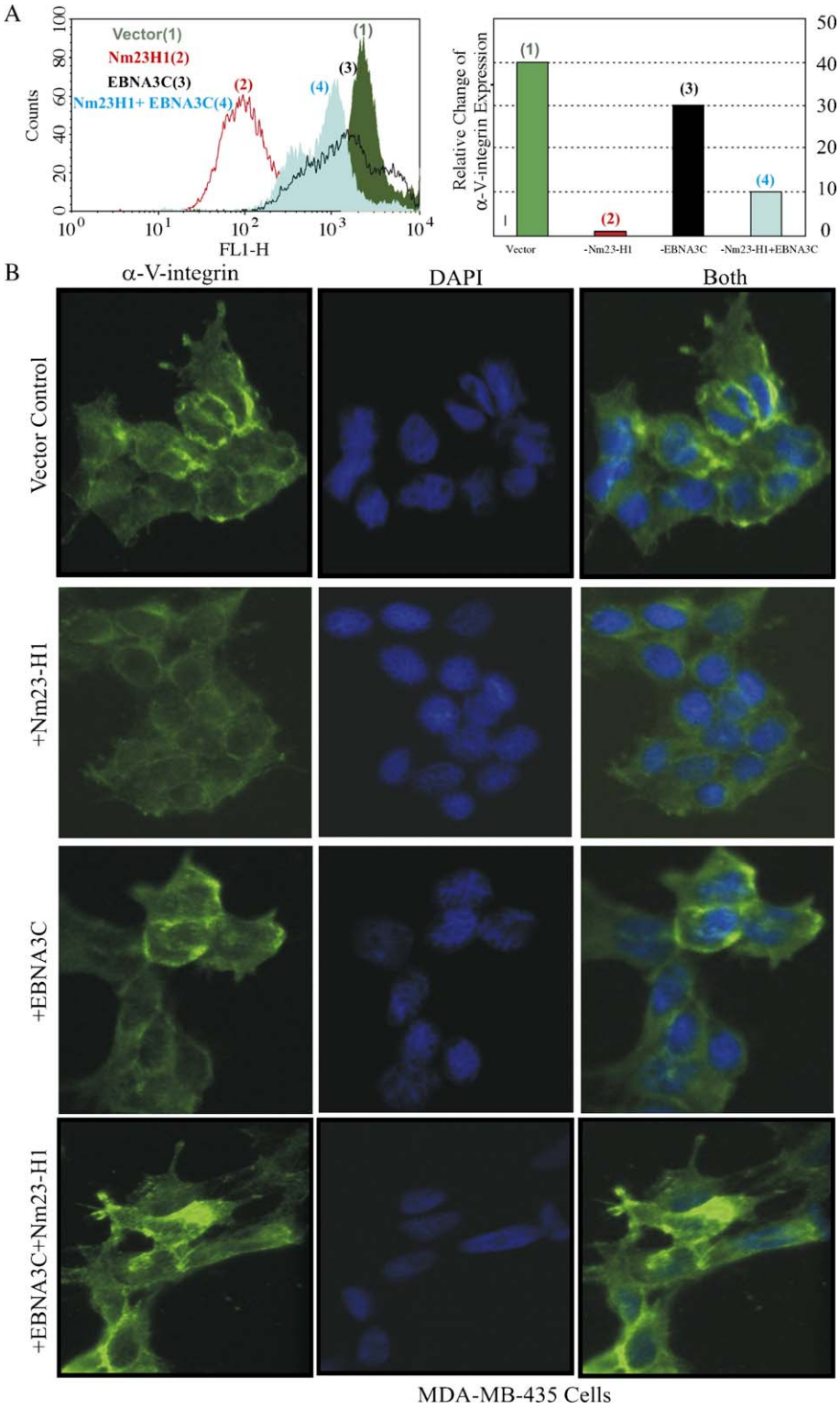


Fig. 2. Alpha V integrin expression level was decreased in Nm23-H1 overexpressed MDA-MB-435 cells. (A) MDA-MB-435 cells stably overexpressing Nm23-H1 as well as expressing EBNA3C or both proteins together were harvested, fixed and stained with alpha-V-integrin-specific mouse monoclonal antibody and further detected by goat anti-mouse secondary antibody conjugated with FITC. Cells were analyzed in FACS Caliber. Data presented in histogram as FL1 in *x* axis. In the right panel, the change of alpha V integrin expression from the FACS data showed in bar diagram. In Nm23-H1 overexpressing cells, the level of alpha V integrin reduced to greater than 40-fold over vector alone control in this assay. (B) MDA-MB-435 cells stably overexpressing Nm23-H1 as well as expressing EBNA3C or both proteins together were grown overnight on cover slip, fixed and stained with alpha-V-integrin-specific mouse monoclonal antibody and further detected by goat anti-mouse conjugate with FITC. Nuclei were stained with DAPI. Immunofluorescence showed reduced cytoplasmic expression of alpha V integrin in Nm23-H1 overexpressing cells.

expression of EBNA3C and Nm23-H1, Western blot analysis was performed, and specific signals were detected using specific antibodies for myc (Nm23-H1 was originally cloned upstream of a myc tag in the pA3M vector) and EBNA3C respectively while the protein levels were determined by β-actin Western blot.

To corroborate the above results, we performed a semi-quantitative real-time PCR analysis. Expression vector pA3M-EBNA3C, pA3M-Nm23-H1 or both were transiently transfected into BJAB and DG-75 cells along with the pA3M vector alone. Real-time PCR for alpha V integrin expression performed as described in Materials and methods. The results show that in Nm23-H1 overexpressing cells the level of endogenous alpha V integrin was reduced to greater than 50-fold over vector alone control in this assay in both cell lines (Fig. 1G). As expected, when EBNA3C was expressed, there was a modest increase in activity (Fig. 1G). Importantly, when EBNA3C and Nm23-H1 were co-expressed, the alpha V integrin transcript levels were at least 2 logs above that seen for Nm23-H1 alone (Fig. 1G, compare lane 2 and 4).

To further support the results of the luciferase reporter assay, FACS and IF analysis were performed. MDA-MB-435 cells stably expressing Nm23-H1 as well as expressing EBNA3C or both proteins together were harvested, fixed and stained with alpha-V-integrin-specific antibody. The flow cytometry data show that in Nm23-H1 overexpressing cells the level of alpha V integrin reduced to greater than 40-fold over vector alone control in this assay (Fig. 2A). In comparison, when EBNA3C was expressed or co-expressed with Nm23-H1, a clear increase in signals above that of Nm23-H1 alone was observed (Fig. 2A). Similarly, experiment done in transiently transfected BJAB cells (Nm23-H1, EBNA3C alone and both protein co-

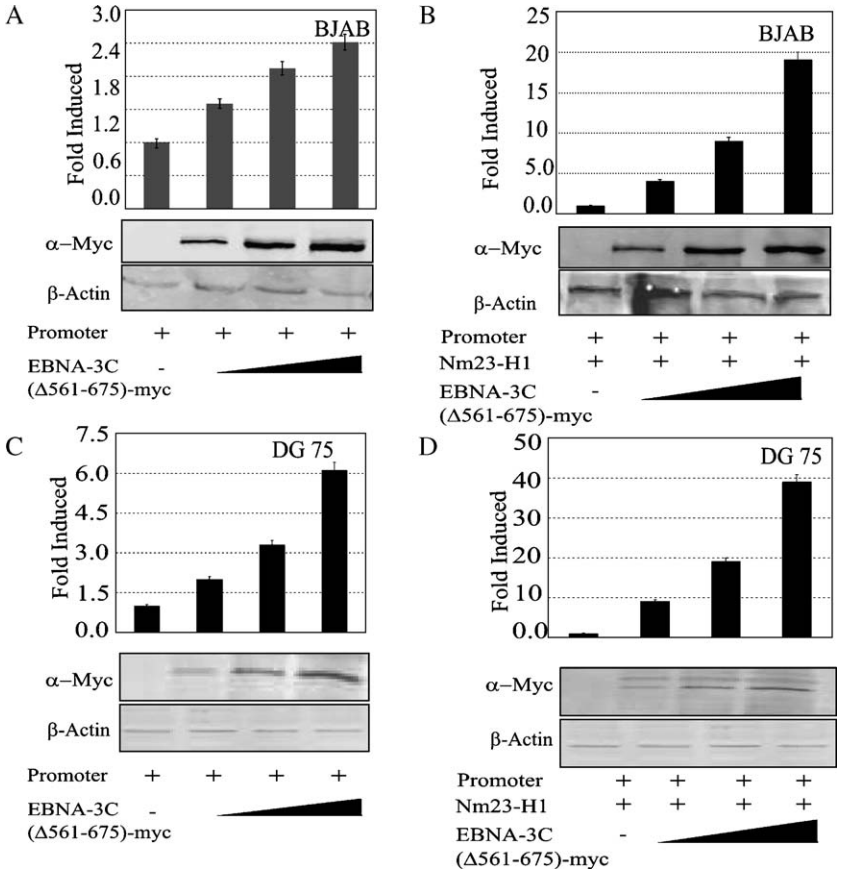


Fig. 3. Modulation of alpha V integrin promoter by Nm23-H1 and EBNA3C is independent to their interaction with each other in BJAB cells. (A) pGL2-alpha V integrin promoter (2.5 μg) was co-transfected with increasing amounts (1 μg, 2 μg, 5 μg) of mutant pA3M-EBNA3C (Δ561–675), and 24 h post-transfection, BJAB cells were harvested for luciferase assay. Increasing amounts of EBNA3C showed a dose-dependent increase in the promoter activity. (B) pGL2-alpha V integrin promoter (2.5 μg) was co-transfected with pA3M-Nm23-H1 (1 μg) and increasing amounts (1 μg, 2 μg, 5 μg) of pA3M-EBNA3C (Δ561–675). Twenty four hours post-transfection, BJAB cells were harvested and lysed for luciferase assay. Increasing amounts of pA3M-EBNA3C (Δ561–675) showed a dose-dependent increase of promoter activity in the presence of constant amount of Nm23-H1. (C) pGL2-alpha V integrin promoter (2.5 μg) was co-transfected with increasing amounts (1 μg, 2 μg, 5 μg) of mutant pA3M-EBNA3C (Δ561–675), and 24 h post-transfection, DG75 cells were harvested for luciferase assay. Increasing amounts of EBNA3C showed a dose-dependent increase in the promoter activity. (D) pGL2-alpha V integrin promoter (2.5 μg) was co-transfected with pA3M-Nm23-H1 (1 μg) and increasing amounts (1 μg, 2 μg, 5 μg) of pA3M-EBNA3C (Δ561–675). Twenty four hours post-transfection, DG75 cells were harvested and lysed for luciferase assay. Increasing amounts of pA3M-EBNA3C (Δ561–675) showed a dose-dependent increase of promoter activity in the presence of constant amount of Nm23-H1. Fractions of lysates were used for Western blot with anti-myc to detect pA3M-EBNA3C (Δ561–675) and pA3M-Nm23-H1. Means and standard deviations were derived from three independent experiments in all the above presented data.

expressed) showed a similar pattern of results as compared to the MDA-MB-435 cell lines (data not shown). In addition, immunofluorescence analysis of the stable cell lines for alpha V integrin suggests a similar pattern based on fluorescent intensity using confocal microscopy (Fig. 2B) corroborating the above FACS data.

Regulation of the of alpha V integrin promoter by Nm23-H1 and EBNA3C is independent of their interaction with each other

Previous studies have shown that the region downstream of aa 365 to 992 of EBNA3C was important in mediating B-cell immortalization (Tomkinson et al., 1993). The metastasis suppressor Nm23-H1 was found to bind to a short stretch of amino acids located between the glutamine- and proline-rich domains of EBNA3C, suggesting an important role for the interaction of Nm23-H1 and EBNA3C (Subramanian et al., 2001, 2002a,b).To determine if EBNA3C and Nm23-H1 can cooperate in regulating the alpha V integrin expression, we used an EBNA3C expression vector deleted for the Nm23-H1 interacting domain (Δ561–675) and a Nm23-H1 expression plasmid that was shown in previous assays to suppress alpha V integrin transcription. The pA3M-EBNA3C (Δ561–675) and pA3M-Nm23-H1 expression plasmids were transiently transfected into BJAB cells along with the luciferase reporter plasmid containing the alpha V integrin promoter. Total transfected DNA was balanced with empty vector, and transfection efficiency was monitored by the use of pGFP and counting of transfected cells for GFP fluorescence. The results showed that the mutant EBNA3C (Δ561–675) moderately activated the alpha V promoter in a dose-dependent manner up to 2.5-fold over vector alone in BJAB cells (Fig. 3A) and 6-fold in DG-75 cells (Fig. 3C). However, the Nm23-H1 induced reduction was reversed to 20- and 40-fold in BJAB and DG75 respectively (Figs. 3B and D). Therefore, the suppression of the alpha V integrin expression by Nm23-H1 can be at least in part reversed by the expression of EBNA3C in this system. However, the revelation of this effect with an EBNA3C molecule which lacks the domain sufficient for binding to Nm23-H1 suggests that this effect is likely to be independent of a direct interaction.

The cellular transcription factor GATA-1 and Sp1 are important for modulation of alpha V integrin promoter

Since neither EBNA3C nor Nm23-H1 has been shown to regulate transcription by directly binding DNA, we looked for possible transcription factors that might be targeted resulting in functional regulation of the alpha V integrin promoter. Sp1 have been previously shown to be transactivator of alpha V integrin expression (Czyz and Cierniewski, 1999). EBNA3C has also been shown to contain a region with homology to the glutamine-rich activation domain of Sp1 which can function as a transcriptional activator when fused to the Gal4 DNA binding domain (Bain et al., 1996). On the other hand, GATA-1 binding to the alpha V promoter negatively regulates expression of the alpha V integrin subunit in human leukemic K562 cells (Czyz et al., 2002). Reporter luciferase plasmids with targeted truncations which remove the Sp1 and GATA-1 binding sites of the alpha V integrin promoter were used in luciferase assays as described above. The results of these assays showed that the reporter constructs deleted for the GATA-1 or Sp1 had little or no apparent change in activity when compared with vector alone control (data not shown). However, when assayed in the context of Nm23-H1 expression, again, a reduction in activity levels was seen (Fig. 4A). Most interestingly, introduction of EBNA3C did not reverse the level of suppression seen by Nm23-H1, suggesting that EBNA3C effect is outside of the region containing the *cis*-acting elements for NF-κb and GATA-1 binding (Fig. 4A). Furthermore, a reporter plasmid deleted for the NF-κb site from −481 to −522 but retaining the element from +169 to −482 was able to reverse the repression by Nm23-H1, when EBNA3C was added to about 75% of the vector alone control and 3-fold over Nm23-H1 (Fig. 4B). Together, these results suggest that NF-κb may not play a role in regulating the level of repression of Nm23-H1 as the levels of repression were more substantial when the NF-κb site was deleted (Fig. 4, compare A and B). To further explore the role of NF-κb, GATA-1 and Sp1 and their binding sites in regulation of alpha V integrin expression in the presence of Nm23-H1 and EBNA3C, we used a construct containing the AP1 and CTC binding site but deleted for other additional sites above (Fig. 4D). The results of the reporter luciferase assay suggest that the majority of the activity in the context of Nm23-H1 and EBNA3C lies with Sp1, NF-κb and GATA-1 as there was minimal changes in the levels seen in the construct with AP1 and CTC site (Fig. 4C). The fact that the changes seen when the NF-κb site was deleted with EBNA3C was almost identical with about 50% of the vector alone indicates that, although NF-κb may be important for the Nm23-H1 effect, it does not play a major role in the ability of EBNA3C to rescue the repression by Nm23-H1.

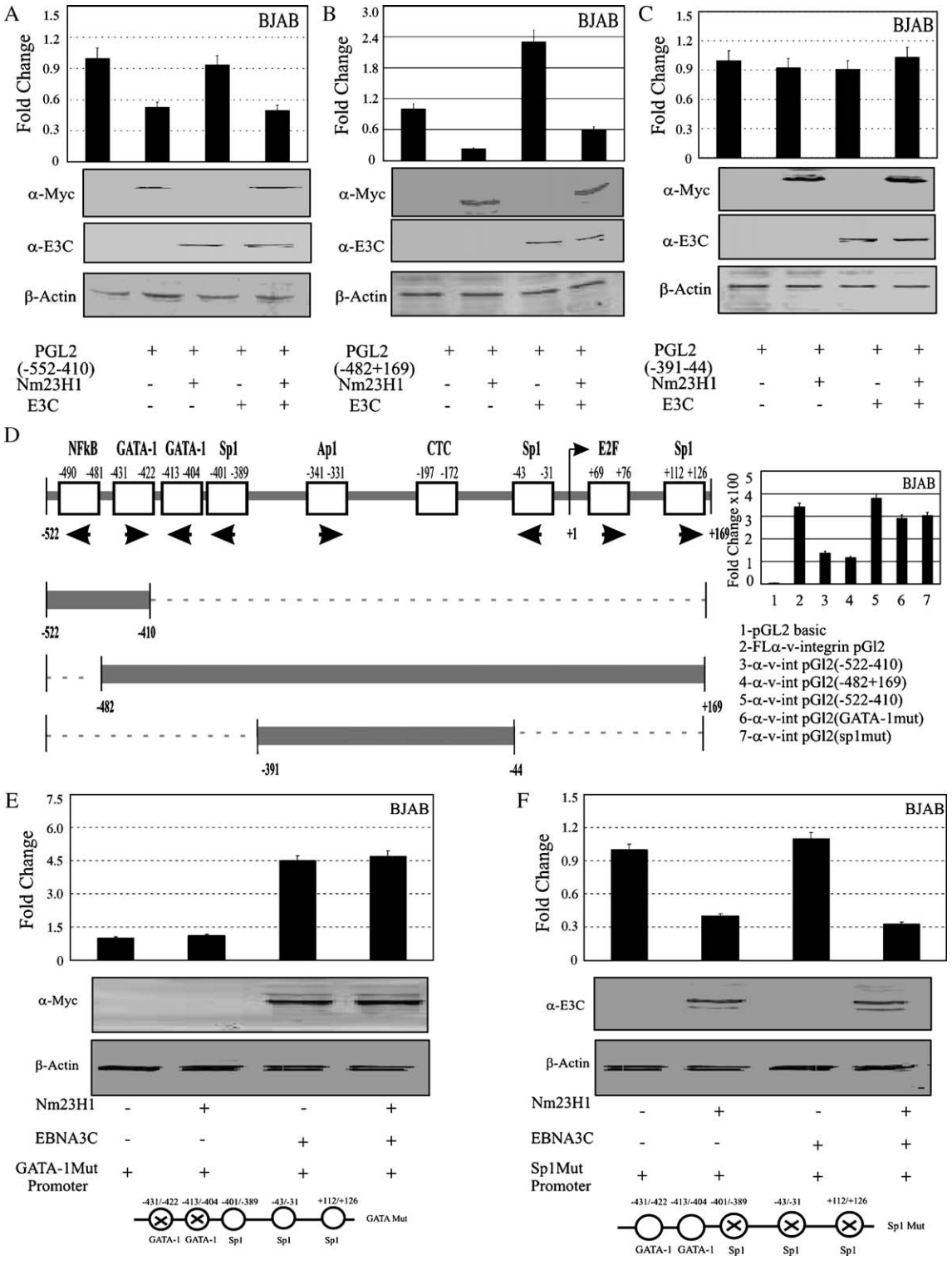
To more specifically address the role of GATA-1 and Sp1, we generate reporter mutants having the two GATA-1 transcription binding site mutated and all the three Sp1 transcription binding site mutated. Studies using the GATA-1 mutant reporter plasmid showed that there was a negligible change in activity in the presence of Nm23-H1 (Fig. 4E). In contrast, the Sp1 mutated reporter plasmid showed about a 50% reduction of activity in the presence of Nm23-H1 (Fig. 4F). Furthermore, we noticed that in Sp1 mutated reporter plasmids there was little or no effect of EBNA3C on the promoter activity (Fig. 4F). These results indicate that the effects of Nm23-H1 and EBNA3C are predominantly through the GATA-1 and Sp1 transcription factors, respectively.

Nm23-H1 forms a complex with the cellular transcription factor GATA-1 in B cells

Data above strongly suggest that Nm23-H1 can down-regulate the expression of alpha V integrin through interactions

with the known cellular transcription factors GATA-1. To demonstrate whether this is through a direct association, EMSA and GST binding experiments were performed using specific antibodies against GATA-1. The results of the luciferase reporter promoter assays indicated that the GATA binding sites within the alpha V integrin promoter are critical for Nm23-H1-mediated downregulation. Therefore, we wanted to determine whether these proteins were regulating alpha V integrins through transcription-factor-mediated DNA binding.

EMSA showed a specific GATA shift observed in BJAB nuclear extract (NE) (Fig. 5). The specificity of this shift was verified through the disappearance of the shift in the presence of specific competitor (Fig. 5, compare lanes 1, 2 and 5). The shift was not disrupted when we used a non-specific cold competitor (Fig. 5, lane 7). The mobility of the GATA-1 probe was reduced



by the NE from BJAB cells in the presence of in vitro translated Nm23-H1 (Fig. 5, lane 3). The presence of Nm23-H1 in the complex was verified by additional supershifting in the presence of anti-myc antibody (1 μg) used to detect Nm23-H1 (Fig. 5, lane 4). BJAB NE where EBNA3C was in vitro translated had no obvious effect on the mobility of the GATA probe at this level of detection (Fig. 5, lane 8). The BJAB NE with mixture of both in vitro translated EBNA3C and Nm23-H1 reduced the mobility of the probe to the same extent as that seen with the Nm23-H1 and BJAB NE alone (Fig. 5, lane 10). There was no apparent change on effect when we used unprogrammed the rabbit reticulocytes (Fig. 5, lane 6). GATA-1-specific antibody (1 μg) showed a specific supershift (Fig. 5, lane 11). Additionally, no specific shift was observed with myc antibody alone (Fig. 5, lane 9). These results suggest that EBNA3C and Nm23-H1 are targeting distinct element within the alpha V integrin promoter and therefore may regulate expression through interaction with different factors bound to the promoter.

A GST-Nm23-H1 pull-down assay was also performed from an NIH3T3 Nuclear extract previously shown to have detectable levels of GATA-1 (Aumont et al., 1993). The results of these binding experiments suggest that Nm23-H1 can associate with GATA-1 in human cell line but not EBNA3C (Fig. 6). The control GST fusion protein alone did not show any specific association with GATA-1 (Fig. 6). Truncated mutants of EBNA3C were used to determine if specific domains of EBNA3C may bind to GATA-1. These mutants were selected from the regions of EBNA3C which include the N-terminal, C-terminal and the region of EBNA3C previously shown to bind to Nm23-H1 (Subramanian and Robertson, 2002). However, the results indicated little or no interaction with GATA-1 (Fig. 6). Therefore, these studies suggest that Nm23-H1 may form a complex with GATA-1 bound to its cognate sequence, down-regulating transcription of the alpha V integrin promoter.

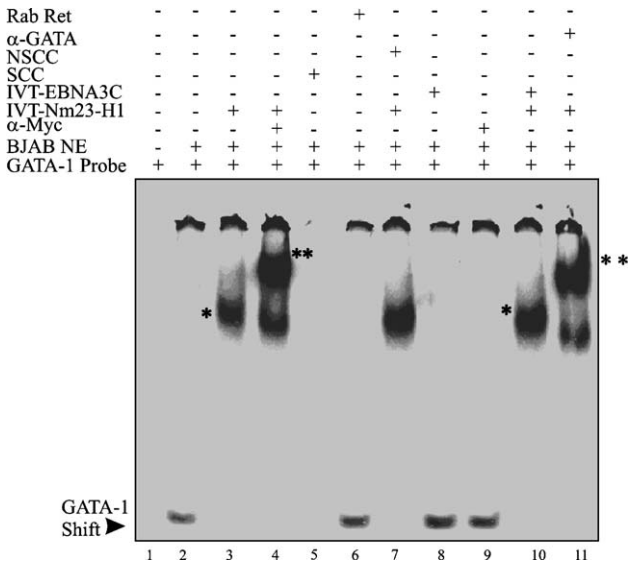


Fig. 5. Nm23-H1 form complexes with GATA-1 transcription factor when it was bound to the cognate sequences demonstrated in EMSA. Probe containing GATA-1 binding site of alpha V integrin promoter along with the flanking bases was labeled with (^{32}P-αdCTP) and used for binding in the presence of in vitro translated Nm23-H1 and EBNA3C. Lanes 1 and 2, probe with and without BJAB nuclear extract (NE). Lane 3 is probe with NE and IVT Nm23-H1 showing shift due to binding of GATA-1 in NE to probe (*). Lanes 5 and 7 are in the presence of specific and non-specific cold competitors, respectively. Lane 4 is showing supershift of the Nm23-H1 and GATA-1 complex in the presence of Nm23-H1-specific antibody (**). Lanes 6, 8 and 9 showing that there were no shifts in the presence of rabbit reticulocytes, IVT EBNA3C and c-*myc* antibody alone, but we observe a shift in the presence of both IVT Nm23-H1 and IVT EBNA3C in lane 10 (*). Lane 11 shows the specific supershift due to GATA-1 specific antibody (**).

EBNA3C associates with the cellular transcription factor Sp1 bound to DNA

The above data strongly suggest that EBNA3C can upregulate the expression of alpha V integrin through interactions with the cellular transcription factor Sp1. To show that this occurs through a direct association with the Sp1 cellular factor, an EMSA experiment was performed using antibodies specific for the Sp1 transcription activators. The results of the luciferase reporter promoter mutants indicated that Sp1 binding sites within the alpha V integrin promoter are critical for EBNA3C-mediated upregulation of alpha V integrin. Therefore, we wanted to test whether these proteins were regulating alpha V integrins through transcription-factor-mediated DNA binding.

Fig. 4. Transcription factors GATA and Sp1 are important for alpha V integrin promoter modulation in BJAB cells. (A) Alpha V integrin promoter region having NF-κB and GATA transcription factor binding sites was modulated by Nm23-H1. 2.5 μg of pGL2-alpha V integrin promoter (−552–410) co-transfected with either 2.5 μg pA3M-Nm23-H1 or 2.5 μg EBNA3C or both protein together. Luciferase assay done at 24 h post-transfection showed decreased alpha V promoter activity in the presence of Nm23-H1. (B) Alpha V integrin promoter region having GATA, Sp1 and AP1 transcription factor binding sites was modulated by EBNA3C and also by Nm23-H1. 2.5 μg of pGL2-alpha V integrin promoter (−482 + 169) was co-transfected either with 2.5 μg pA3M-Nm23-H1 or 2.5 μg EBNA3C or both proteins. Twenty four hours post-transfection, the harvested cells showed an increase in the promoter activity. In contrast to that, promoter activity was sharply decreased in the presence of Nm23-H1. Decrease of expression was also observed in the presence of both proteins. (C) Alpha V integrin promoter containing only AP1 binding site did not modulate by either EBNA3C or Nm23-H1. 2.5 μg of pGL2-alpha V integrin promoter (−391–44) was co-transfected either with 2.5 μg pA3M-Nm23-H1 or 2.5 μg EBNA3C and both protein. Twenty four hours post-transfection, cells were harvested and lysed for luciferase assay. No significant change was observed in the presence of either of the proteins. (D) Schematic of alpha V integrin promoter indicating the transcription factor binding sites along with the truncated/deletion mutant used in the above assay. (E) Alpha V integrin promoter not having GATA-1 binding site did not modulate by Nm23-H1. 2.5 μg of pGL2-alpha V integrin promoter (GATA-1 mutant) was co-transfected either with 2.5 μg pA3M-Nm23-H1 or 2.5 μg EBNA3C and both protein. Twenty four hours post-transfection, cells were harvested and lysed for luciferase assay. No significant change was observed in the presence of Nm23-H1 but a significant change in the presence of EBNA3C. (F) Alpha V integrin promoter not having Sp1 binding site did not modulate by EBNA3C. 2.5 μg of pGL2-alpha V integrin promoter (Sp1 mutant) was co-transfected either with 2.5 μg pA3M-Nm23-H1 or 2.5 μg EBNA3C and both protein. Twenty four hours post-transfection, cells were harvested and lysed for luciferase assay. No significant change was observed in the presence of EBNA3C but a significant change in the presence of Nm23-H1. Fractions of lysates were used to detected proteins by Western blot. Means and standard deviations were derived from three independent experiments in all the above presented data.

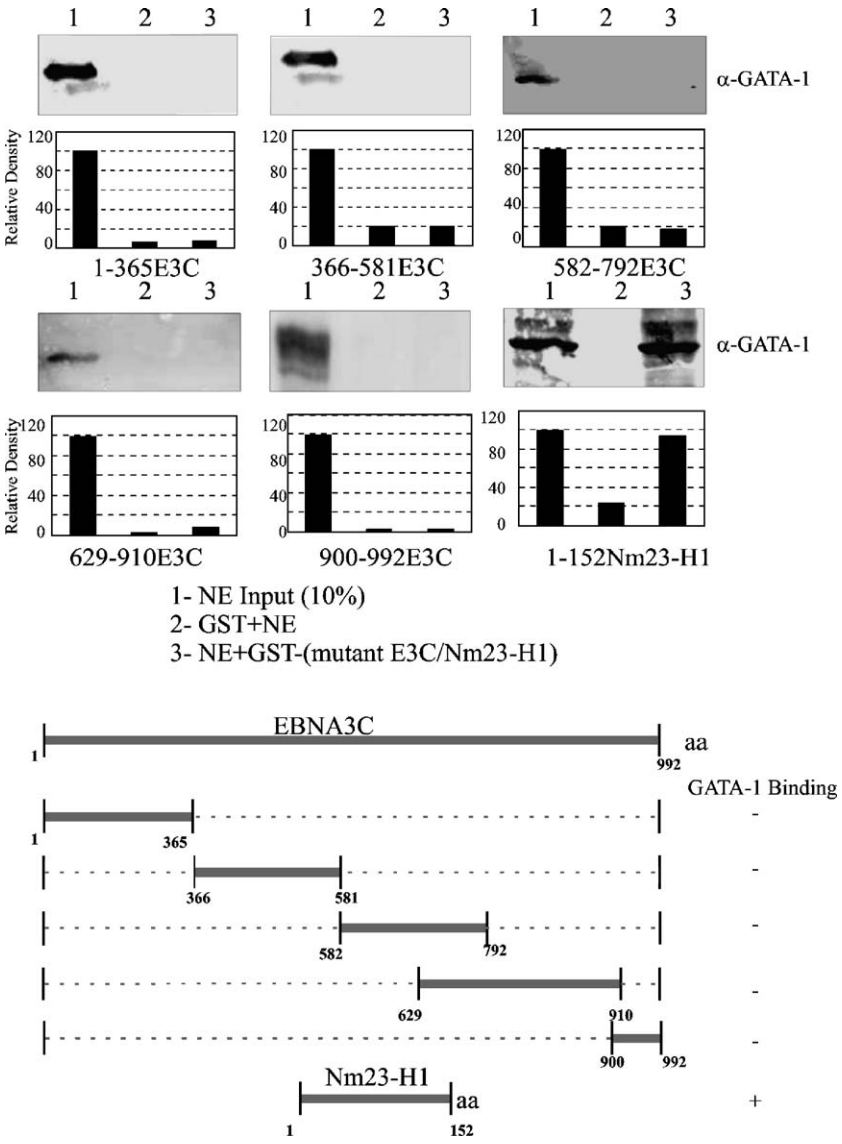


Fig. 6. Nm23-H1 but not EBNA3C truncated mutants bind with GATA-1 in NIH3T3 nuclear extract (NE). NIH3T3 nuclear extract incubated with GST and Nm23-H1 GST along with the mutants E3C-GST (1–365, 366–581, 582–792, 629–910, 900–992) protein for 3 h. Bound protein was resolved on 12% SDS-PAGE, transferred and detected using anti-GATA (rat monoclonal) antibody. Relative bindings are shown in bar diagram.

The results showed a specific Sp1 shift observed in BJAB nuclear extract (Fig. 7). The specificity of the shift was verified by its disappearance in the presence of cold specific competitor (Fig. 7, compare lanes 1, 2 and 5). The shift was not lost when a non-specific cold competitor was used (Fig. 5, lane 6). The mobility of the Sp1 probe was reduced by the NE from BJAB cells and further in the presence of in vitro translated EBNA3C (Fig. 7, lane 3). The presence of EBNA3C in the complex was further verified by additional supershifting in the presence of a specific anti-EBNA3C (myc ascites, 1 μg) antibody (Fig. 7, lane 4). BJAB NE where Nm23-H1 was in vitro translated had no obvious effect on the mobility of the Sp1 probe at this level of detection (Fig. 7, lane 8). The BJAB NE and mixture both in vitro translated EBNA3C and Nm23-H1 reduced the mobility of the probe to the same extent as that seen with the EBNA3C and BJAB NE alone (Fig. 7, lane 10). These results suggest that EBNA3C and Nm23-H1 are binding to factors at different positions of the alpha V integrin promoter to regulate expression. The unprogrammed rabbit reticulocyte had no effect (Fig. 7, lane 11). However, the Sp1-specific antibody (1 μg) supershifted the complex (Fig. 7, lane 9). Additionally, no specific shifts were observed with antibody alone (Fig. 7, lane 7). These results strongly suggest that EBNA3C can associate with the transcription activators Sp1 mediating upregulation of alpha V integrin transcription.

Nm23-H1 downregulates the autophosphorylation of FAK at Tyr^{397}

Focal adhesion kinase (FAK) is a central player in the control of the cell migration by activating autophosphorylation at Tyr^{397} (Cooper et al., 2002; Hynes, 1992). Additionally, αVβ3 ligation

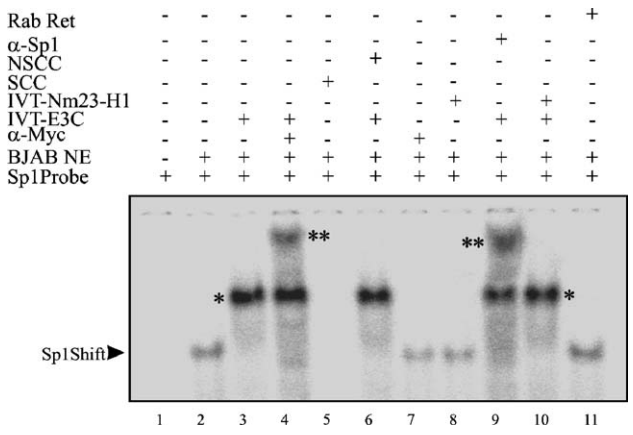


Fig. 7. EBNA3C form complexes with Sp1 transcription factor to its cognate sequences. A probe for Sp1 (GC Box) binding site of alpha V integrin promoter along with the flanking bases was labeled with (^{32}P-αdCTP) and used for binding in the presence of in vitro translated Nm23-H1 and EBNA3C. Lanes 1 and 2, probe with and without BJAB nuclear extract (NE). Lane 3 is probe with NE and IVT EBNA3C showing shift due to binding to Sp1 (*). Lanes 5 and 6 are with specific and non-specific cold competitors along with probe, NE and IVT EBNA3C myc tagged. Lane 4 shows the supershift of EBNA3C and Sp1 complex in the presence of anti-myc (**) antibody. Lanes 11, 8 and 7 containing rabbit reticulocytes, IVT Nm23-H1 and anti-myc alone, respectively, had no affect on probe mobility, whereas the presence of both IVT Nm23-H1 and IVT EBNA3C in lane 10 showed shift in the mobility (*). Lane 9 shows supershift in the presence of Sp1-specific antibody (**).

to extracellular matrix (ECM; mainly the vitronectin component) activates FAK. We used the breast cancer cell line MDA-MB-435 stably transfected with EBNA3C, Nm23-H1 and both constructs along with the empty vector control so as to have consistent expression with majority of cells compared to transient expression. Upon inducing αVβ3 with vitronectin for 24 h, we analyzed the level of autophosphorylation along with the basal level of FAK by Western blot and in cell Western analysis.

Our Western blot results showed that there was no change in expression of basal level of FAK in the stable cell lines including the control MDA-MB-435 cells treated with the specific inhibitor of αVβ3 receptor (Tucker, 2003). Interestingly, we observed a 3-fold reduction of autophosphorylation at FAK-Tyr^{397} in the cell expressing Nm23-H1 (Fig. 8A). A similar trend in activity is seen as observed by the in cell Western study (Fig. 8B) when comparing the results in the presence of the specific inhibitor LM609 (Fig. 8B, compare lane 2 and 5).

Discussion

EBV transformation of B-lymphocytes requires a subset of six latent proteins including EBNA3C (Subramanian et al., 2002a,b). EBNA3C has been shown to function as a transcriptional activator or repressor depending on its interaction with specific cellular factors (Subramanian et al., 2002a,b). Previous studies have shown that EBNA3C can also repress the major LMP1 latent promoter in large part through its interaction with the RBP-Jκ transcription repressor, through disruption of the EBNA2/RBP-Jκ interaction as well as the interaction of RBP-Jκ with its cognate sequence within the LMP1 promoter (Robertson et al., 1995, 1996; Marshall and Sample, 1995). In this report, we show interactions between EBNA3C and the cellular transcription factor Sp1.

The interaction between EBNA3C and Nm23-H1 and resulting downstream effects is important to understand the mechanism by which this essential viral oncoprotein and the known cellular metastasis suppressor can regulate cell proliferation and cell migration in vitro in EBV-positive tumors (Subramanian et al., 2001). The region of EBNA3C which binds Nm23-H1 has been mapped to a 39-aa carboxy-terminal

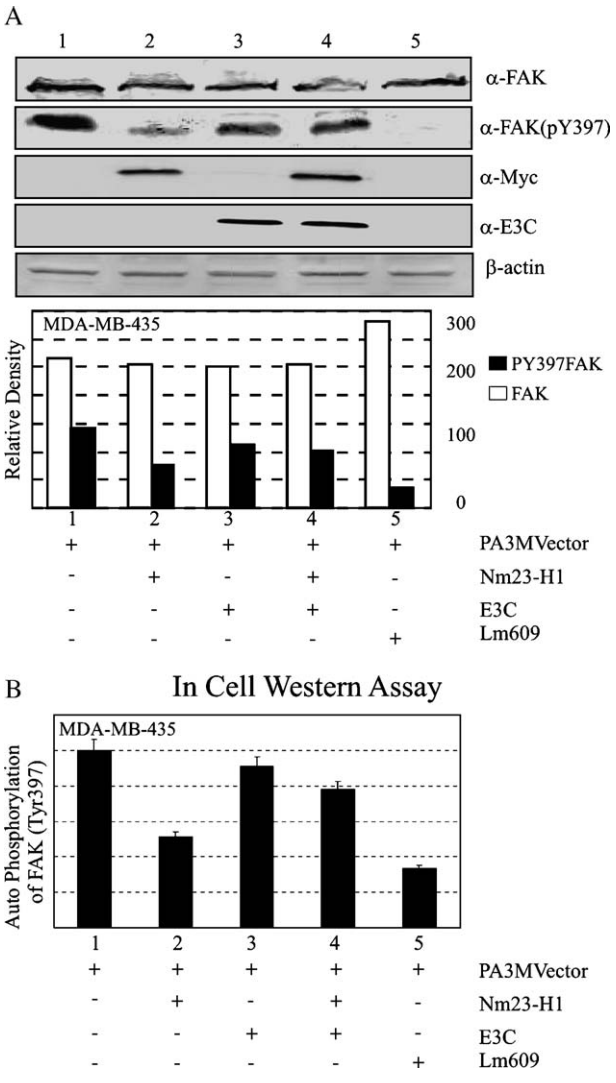


Fig. 8. Autophosphorylation of FAK in the presence of Nm23-H1 and EBNA3C. (A) Lysates of MDA-MB-435 cells grown in the presence of vitronectin vector control (1), stably transfected with Nm23-H1 (2), stably transfected with E3C (3), stably co-transfected with Nm23-H1 and E3C (4) and vector control in the presence of LM609 (5) were immunoblotted with rabbit polyclonal anti-FAK and mouse monoclonal anti-Phospho-FAK (Y397) and then normalized with β-actin. (B) In cell Western, MDA-MB-435 cells grown in the presence of vitronectin vector control (1), stably transfected with Nm23-H1 (2), stably transfected with E3C (3), stably co-transfected with Nm23-H1 and E3C (4) and vector control in the presence of LM609 (5) and presented autophosphorylation with respect to the basal level of FAK. Means and standard deviations were derived from three independent experiments.

sequence located between the glutamine- and proline-rich domains of the protein (Subramanian and Robertson, 2002). The location of this binding site is interesting because the glutamine-rich region is known to function as a transactivation domain, suggesting that its interaction with Nm23-H1 may affect the ability of EBNA3C to regulate transcription. Moreover, it has previously been shown that EBNA3C can increase Nm23-H1-mediated activation of a basal promoter, and this effect is dependent on the glutamine-rich region since the Nm23-H1 binding domain alone had little effect on activation (Subramanian and Robertson, 2002). Thus, the interaction domain may serve as a recruitment domain for the glutamine-rich functional activator of EBNA3C.

The mechanism by which EBNA3C is able to reverse the cell migratory effects of Nm23-H1 is not known, though it has been suggested that it may in part be due to upregulation of adhesion molecules E-cadherin and α-integrins (Subramanian et al., 2001). There are also reports indicating the possible role of MMP-9 (Kuppers et al., 2005). Additionally, it is also possible that other functional effects of Nm23-H1 may be affected by EBNA3C. Here, in this report, we specifically focused on alpha V integrin. Alpha V integrin is one of the major proteins reported to be a key player in cancer development and progression (Cooper et al., 2002; McGary et al., 2002; Weber, 2001; Ruoslahti, 1996; Cheresh, 1991). Here, we report an observed increase in alpha V integrin expression in the presence of EBNA3C. In contrast, we observed a decrease in expression in the presence of Nm23-H1. Thus, these two proteins can counteract the effect of each other at least in terms of alpha V integrin expression. In the line of counteractive theory, these proteins may also antagonize each other either by direct interaction through a large complex or through the effects of independent complex. Thus, a possible mechanism of by which EBNA3C can induce cell migration and possibly metastasis is by regulating the alpha V integrin signaling pathway.

Promoter studies of alpha V integrins give us an indication that Nm23-H1 clearly downregulated the promoter in two different B-cell lines. The semi-quantitative real-time PCR analysis also supported these conclusions. These data were further supported by the FACS and IF findings which show that there was a definite decrease of expression of alpha V integrin in MDA-MB-435 cells with stably expressing Nm23-H1. Deletion- and site-specific mutational analysis of the alpha V integrin promoter revealed that GATA-1 is a critical component for Nm23-H1-mediated downregulation while Sp1 has a more pronounced role in EBNA3C-mediated upregulation. Analysis of the specific GATA-1 and Sp1 mutants of alpha V integrin promoter confirmed our hypothesis. The results of this transient reporter studies were further supported by EMSA and GST binding results, from BJAB and NIH3T3 cells which demonstrated the same general trend as the reporter assay. These studies corroborated the finding that both Nm23-H1 and EBNA3C can counter the effect of each other by direct association or indirectly through association with other known transcription activators to regulate the alpha V integrin promoter.

Nm23-H1 has been shown to localize to the cytoplasm; however, in the presence of EBNA3C, its localization was shown to shift to an almost exclusively nuclear signal (Subramanian et al., 2001). This change in localization strengthens the possibility that Nm23-H1 may function as a transcriptional regulator. However, in uninfected cells, low levels have been detected in the nucleus, suggesting that a cellular partner may play a role in this translocation or that Nm23-H1 itself maybe post-translationally modified to mediate this event. Unpublished data in our laboratory have shown that Nm23-H1 can bind to Nm23-H2 (Choudhuri and Robertson, unpublished data), the predominantly nuclear family member supporting a potential role for Nm23-H1 in the nucleus independently or in association with Nm23-H2.

Based on our promoter analyses data and GST results, we have determined that Nm23-H1 and EBNA3C bind to the cellular transcription activators GATA-1 and Sp1, respectively. Additionally, gel shift study suggests that these associations are important for the regulation of the alpha V integrin promoter. This finding is the first report of the association of Nm23-H1 with cellular transcription factor GATA-1. The reporter analyses provide strong evidence that Nm23-H1 is capable of regulating cellular proteins through its interaction with transcription activators bound to their responsive elements within the promoter of the target molecules. It has also been suggested that Nm23-H1 may directly bind DNA as reported, showing repression of transcriptional activity of the platelet-derived growth factor-A (Ma et al., 2002). Additionally, another report showed that alpha V integrin is negatively regulated by the GATA-1 (Czyz and Cierniewski, 1999). This study has been further confirmed by data from EMSA and also by the in vivo GST binding. However, we have not been able to show that Nm23-H1 directly bound to DNA in our studies after multiple attempts. Additional studies are required to elucidate this potential dual effect on transcriptional regulation mediated by Nm23-H1.

The EMSA data provide convincing evidence that EBNA3C and Nm23-H1 can act independently to regulate alpha V integrin primarily through the GATA-1 and Sp1 transcription factors respectively bound to the alpha V integrin promoter. Translocation of Nm23-H1 to the nucleus followed by binding to GATA-1 bound to its cognate sequence can negatively regulate the promoter activity. However, it should be noted that GATA-1 was previously shown to negatively control the alpha V integrin promoter (Czyz et al., 2002). Moreover, the same study showed that the Sp1 binding site located within the alpha V integrin promoter can influence upregulation of the expression (Czyz and Cierniewski, 1999). Our EMSA data show that both Nm23-H1 and EBNA3C can bind to GATA-1 and Sp1, respectively, complexed to their cognate sequences thus regulating expression. In addition, EBNA3C and Nm23-H1 may interact with each other in omplex with GATA-1 and Sp1 at the promoter, thus leading to activation of transcription.

Furthermore, we also showed that Nm23-H1 was able to downregulate one of the downstream factors of the αVβ3 signaling pathways (Parsons, 2003). The downregulation of autophosphorylation in FAK at Y397 by Nm23-H1 was dramatically reversed in the presence of EBNA3C possibly through translocation of Nm23-H1 to the nucleus (see model in Fig. 9).

The role of EBV in metastasis is clearly very complex, given the difference in viral protein expression across the latency programs established by EBV. However, the potential for regulation of metastasis does exist with the viral-associated cancers (Rickinson and Kieff, 1996). The overall role of EBNA3C in this process may only be one aspect of this complicated regulatory pathway in that its expression so far has been detected mostly in type III latency (Young and Murray, 2003; Rickinson and Kieff, 1996). Only a few known EBV-associated cancers are associated with the latency III program, and they are predominantly seen in immunocompromised patients (Rickinson and Kieff, 1996). However, the expression of EBNA3C during the initial or early stages of infection in other positive cancers is possible and may shed light on its potential role in regulating genes involved in cell migration (Subramanian et al., 2001). In addition, the prevalence of HIV and transplants-associated lymphomas continues to rise; therefore, the importance of EBNA3C in EBV-associated malignancies is also expected to increase as the incidence of these cancers increases (Fig. 9).

Materials and methods

Cell lines and antibodies and constructs

BJAB and DG75 cells are EBV-negative B cells isolated from Burkitt's lymphoma patients and were provided by Elliott Kieff (Brigham and Womens Hospital, Boston, MA). B-cell lines were grown in RPMI 1640 medium (Hyclone, Logan, UT) supplemented with 10% bovine growth serum, 2 mM glutamine and 25 U/ml penicillin/streptomycin. Human breast cancer cells MDA-MB-435 were maintained in DMEM (Hyclone, Logan, UT) supplemented with 10% bovine growth serum, 2 mM glutamine and 25 U/ml penicillin/streptomycin.

A rat monoclonal antibody against GATA-1 (E-3) and a rabbit polyclonal antibody against alpha V integrin (sc-52) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Polyclonal rabbit anti-FAK (556368) and mouse monoclonal FAK-pY397 (611806) were purchased from BD Biosciences (Beverly, MA). The specific inhibitor of αVβ3 mouse monoclonal antibody was obtained from Chemicon (Temecula, CA). A10 monoclonal reactive to EBNA3C and anti-myc ascites reactive to myc has been previously described Knight et al. (2001).

The alpha V integrin reporter plasmid was provided by Glen Nemerow (Scripps Research Institute, La Jolla, CA), and the specific truncations were sub-cloned in the lab in pGL2 basic plasmid. The alpha V integrin promoter with deleted GATA-1 and deleted Sp1 transcription sites was generated by PCR-based site mutagenesis.

Transfection

BJAB, DG-75 and MDA-MB-435 cells were transfected by electroporation using a BioRad Gene Pulser II electroporator. Ten million cells were collected and washed once in phosphate-buffered saline. The cells were then resuspended in 400 μl of either DMEM or RPMI 1640 containing DNA normalized to balance total DNA, and transfection efficiency was determined by GFP expression as an internal control. Once resuspended, the cells were transferred to 0.4 cm electroporation cuvettes and electroporated at 975 μF and 220 V for BJAB, DG-75 and MDA-MB-435 cells. Following electroporation, the cells were plated in 10 ml of supplemented

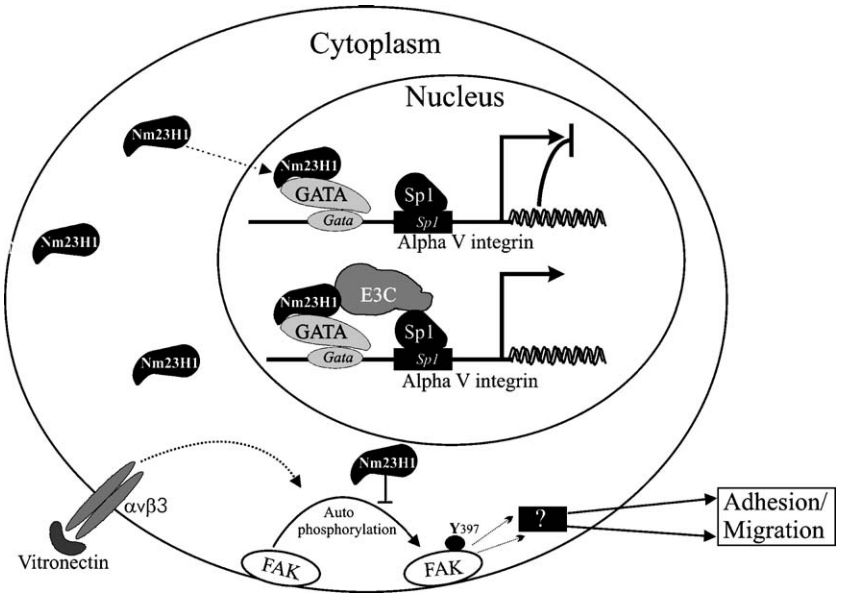


Fig. 9. Hypothetical model for the regulation of alpha V integrin promoter by EBNA3C and Nm23-H1. Regulation of alpha V integrin by Nm23-H1 and EBNA3C is possibly independent to their interaction with each other. Downregulation of the alpha V integrin activity by Nm23-H1 is mediated through binding to GATA-1. In contrast, EBNA3C upregulates alpha V integrin expression through binding to Sp1 transcription factor. Nm23-H1 also inhibits the autophosphorylation of focal adhesion kinase (FAK) at Tyr^{397} and thus blocks the αvβ3 pathway.

media and grown at 37 °C with 5% CO_2 for 20 h before being harvested.

Luciferase assay

Ten million BJAB, DG-75 and MDA-MB-435 cells were collected at a concentration of 5×10^5/ml and transfected as described above. Twenty hours post-transfection, the cells were harvested, washed in PBS and lysed in 400 μl of Reporter Lysis Buffer (Promega, Inc., Madison, WI). A 40-μl aliquot of the lysate was then mixed with 100 μl of luciferase assay reagent in an Opticomp Luminometer (MGM Instruments, Inc., Hamden, CT), and luminescence was read for 10 s. Diluted lysates were also measured to ensure that the values were within the linear range of the assay. The results presented are the mean of experiments performed in triplicate.

Western blotting

Western blotting assays were performed as previously reported (Lan et al., 2004). Western blots were performed using antibodies specific to EBNA3C, Nm23-H1, and fluorescence-labeled secondary antibodies (Rockland, Inc., Gilbertsville, PA) followed by detection with an Odyssey Imager (LiCor, Inc., Lincoln, NE).

Real-time quantitative PCR

Total RNA from 24 h post-transfected cells (vector only, Nm23-H1, EBNA3C and both Nm23-H1 and EBNA3C) DG75 and BJAB cell lines were isolated using Trizol reagent (Invitrogen, Inc., Carlsbad, CA) following the manufacturer's instructions. cDNA was made using a Superscript II RT kit (Invitrogen, Inc., Carlsbad, CA) according to the manufacturer's instructions. The specific primers for alpha V integrin used were as follows: sense, 5′-TGTGCAGCCAATACCCATCTCAAT-3′; antisense 5′-CGTTCAAACCAGCCAACCAACA-3′ which yielded a 252-bp PCR product. β-actin was amplified by using the primers: sense, 5′-GCTCGTCGTCGACAACGGCTC-3′, antisense, 5′-CAAACATGATCTGGGTCATCTTCTC-3′. This yielded a 352-bp PCR product. The target gene was amplified from cDNA using SYBR green real-time master mix (MJ Research Inc., Waltham, MA), 1 mM each primer and 1 μl of the cDNA product in a total volume of 20 μl. Thirty-five cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, followed by 10 min at 72 °C, were performed in an MJ Research Opticon II thermocycler (MJ Research Inc., Waltham, MA). Each cycle was followed by two plate reads, with the first at 72 °C and the second at 85 °C. A melting curve analysis was performed to verify the specificity of the products, and the values for the relative quantitation were calculated by the $\Delta\Delta C_t$ method. The experiment was performed in triplicate.

GST fusion protein preparation, nuclear lysate binding assays

BL21 (*E. coli*) cells were transformed with pGex2TK-Nm23-H1 plasmid and selected on ampicillin plate. Overnight grown culture from a single colony was inoculated into 500 ml of LB medium and grown to mid-exponential phase with shaking. The cells were then induced with 1 mM isopropyl-B-D-thiogalactopyranoside (IPTG) overnight at 30 °C with shaking. The cells were subsequently harvested, sonicated and the protein was solubilized. The lysate was then incubated with Glutathione Sepharose beads overnight at 4 °C with rotation. The beads were collected by centrifugation and then washed four times with NETN (20 mM Tris–HCl (pH 8.0)/100 mM NaCl/1 mM EDTA/0.5% Nonidet P-40) containing protease inhibitors. The protein-bound beads were stored at 4 °C in NETN containing protease inhibitors.

The NIH3T3 Nuclear Extract used in the binding experiments was prepared as described previously (Knight et al., 2001). The nuclear extract was then precleared with Glutathione Sepharose beads for 30 min at 4 °C with rotation. The lysates were additionally precleared with GST-bound Glutathione Sepharose beads for 1 h at 4 °C with rotation. The lysates were then incubated with an amount of GST-Nm23-H1 bound beads, equivalent to the GST bound beads used to preclear and rotated overnight at 4 °C. SDS lysis buffer with heating was used to elute the bound protein from the beads followed by 10% SDS-PAGE. Western blots using anti-GATA-1 antibody were used to detect the transcription GATA-1 factor associated with Nm23-H1.

Electrophoresis mobility shift assays (EMSA)

The probes for the GATA-1 and Sp1 binding sites within the alpha V integrin promoter have been previously described (Czyz and Cierniewski, 1999; Czyz et al., 2002; Knight et al., 2001). The probes were end-labeled by Klenow fill-in reaction with [α-^{32}P] dCTP and purified with NucTrap Probe Purification Columns (Stratagene, Inc., La Jolla, CA). Radioactive probes were diluted in STE (100 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA) to a final concentration of 100,000 cpm/μl. DNA binding reactions and the preparation of nuclear extracts were performed as described previously (Knight et al., 2001). BJAB cells were used for preparing nuclear extract. Fifteen micrograms of protein from nuclear extracts was mixed with 1 μg poly (dI–dC) (Sigma) in DNA binding buffer (20 mM HEPES pH 7.5, 0.01% NP-40, 5.0% glycerol, 10 mM $MgCl_2$, 100 μg of bovine serum albumin, 1 mM DTT, 1 mM PMSF, 40 mM KCl) to a total volume of 50 μl and incubated at room temperature for 5 min. One microliter of labeled probe was added to each reaction followed by an additional 15 min at room temperature. Cold competitors (200×) were added prior to the initial incubation at room temperature. Rabbit polyclonal antibodies against Sp1 and rat monoclonal antibodies of GATA-1 as well as A10 mouse monoclonal against EBNA3C and anti-myc ascites were used to supershifting the specific bands. DNA–protein complexes were resolved on a non-denaturing 6% PAGE run in 0.5× TBE buffer at a constant voltage of 150 V. Following electrophoresis, the gels were dried and exposed to a Phosphor Imager Screen (Amersham Biosciences, Piscataway, NJ) for 48–72 h.

Flow cytometric analysis of alpha V integrins expressions at protein level

For the determination of the expression of alpha V integrin protein, 5 million MDA-MB-435 cells from stable cell line (vector only, Nm23-H1, EBNA3C and both Nm23-H1 and EBNA3C) were fixed in methanol and then permeabilized. Cells from each group were incubated with polyclonal anti-alpha V integrin (2 mg/ml in PBS containing 1% BSA) for 2 h at room temperature and then with FITC-conjugated isotype-specific secondary antibody for detecting the respective protein levels for 1 h. Cells were washed thoroughly and analyzed on a flow cytometer (Becton Dickinson, San Diego, CA) equipped with 488 nm Argon laser light source and a 623 nm band pass filter for FITC fluorescence. A total of 10,000 events were acquired for analysis using CellQuest software (Becton Dickinson, San Diego, CA). Cells were properly gated as described earlier, and histogram plot of FITC fluorescence (x axis) versus counts (y axis) has been shown in logarithmic fluorescence intensity.

Immunofluorescence

Immunofluorescence assays were performed essentially as described previously (Subramanian et al., 2001; Stamenkovic, 2000). Briefly, fixed cells were blocked in the appropriate serum and then incubated with the specific primary antibody for alpha V integrins for 1 h. Cells were washed and then further incubated with the appropriate secondary antibody conjugated to Alexa Flour 488 at 1:1000 dilutions in phosphate-buffered saline for 1 h. Slides were washed, visualized with an Olympus Flouview 300 IX81 inverted confocal microscope, and photographed with a digital camera and Flouview software (Olympus, Melville, NY).

In cell Western

We used in cell Western analysis for determination of autophosphorylation of focal adhesion kinase, a downstream effector of $\alpha v\beta 3$. MDA-MB-435 cells were stably transfected with EBNA3C, Nm23-H1 and both along with the empty vector control grown up to 80% confluency in T75 flask. The cells were collected and diluted to 10^5 cells/ml in serum free media. Two hundred microliters of cell suspension was dispensed in 96-well plate (20,000 cells/well) coated with vitronectin (0.2 μg/cm^2). At the same time, the inhibitor of vitronectin LM609 (25 μg/ml, treated for 24 h) was used as a negative control. Cells were fixed with 4% formaldehyde for 20 min after thorough washing with 1× TBS. This is followed by permeabilization and washing with 0.1% Triton X-100 in 1× TBS. Cells were then incubated with both FAK (rabbit polyclonal, 1:1000) and Phospho-FAK (mouse monoclonal 1:1000) antibody overnight. Secondary antibodies Goat anti-rabbit IRDye 800 and Goat anti-mouse IR Dye 680 (Molecular Probes, Inc) were used (1:800) and further incubated for 1 h. Plates were then dried in the dark and scanned for detection in both the 700 and 800 channels using Odyssey infrared scanning system.

Acknowledgments

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