CLONING OF THE HUMAN ACTIVATED LEUKOCYTE CELL ADHESION MOLECULE PROMOTER AND IDENTIFICATION OF ITS TISSUE-INDEPENDENT TRANSCRIPTIONAL ACTIVATION BY Sp1

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Abstract: Activated leukocyte cell adhesion molecule (ALCAM) belongs to the immunoglobulin cell adhesion molecule super family. ALCAM is implicated in tumor progression, inflammation, and the differentiation of hematopoietic stem cells. Hitherto, the identity of regulatory DNA elements and cognate transcription factors responsible for ALCAM gene expression remained unknown. In this report, the human ALCAM promoter was cloned and its transcriptional mechanisms elucidated. The promoter is TATA-less and contains multiple GC-boxes. A proximal 650-bp promoter fragment conferred tissue-independent activation, whereas two contiguous regions upstream of this region negatively influenced promoter activity in a tissue-specific manner. The positive regulatory promoter region was mapped to a core 50 base pair sequence containing a conical Sp1 element. Mutation analysis revealed that this element alone or in tandem with elements immediately upstream was required for maximal promoter activity. Chromatin analysis revealed that Sp1 binds exclusively to the canonical binding sequence in vivo, but not to DNA sequence immediately upstream. Finally, we showed that over-expression of Sp1 significantly increased the basal promoter activity. Thus, Sp1 activated the

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Abbreviations used: ALCAM – activated leukocyte cell adhesion molecule; CHIP – chromatin immunoprecipitation; EMSA – electrophoretic mobility shift assay; PAECs – pulmon. artery endothelial cells; PMVECs – pulmon. micro-vascular endothelial cells; pN3Sp1 – plasmid DNA expressing Sp1; Sp1 – specificity protein 1; TBP – TATA-box binding protein; TFIIB – transcription factor IIB; TRANSFAC – TRANScription FACTor database
ALCAM promoter in most cells. These findings have important ramifications for unraveling the roles of ALCAM in inflammation and tumorigenesis.

Key words: ALCAM, Cis elements, Endothelial cells, Epithelial cells, Hematopoietic cells, Promoter activity, Sp1, Transcriptional regulation

INTRODUCTION

The gene for activated leukocyte cell adhesion molecule (ALCAM) is located on the long arm of human chromosome 3 [1]. It encodes a protein belonging to the immunoglobulin cell adhesion superfamily that engages in homotypic and heterotypic adhesions, the latter being with the T-lymphocyte co-stimulatory molecule CD6 [1-3]. ALCAM is known by several other names including BEN [4, 5], neurolin [6], DM-GRASP [7], HCA [8, 9], MEMD [10], HB2 [11], SC1 [12] and CD166 [13]. It is transiently expressed in human blastocysts and endometrial epithelial during embryonic implantation [14], and its transcripts are present in the para-aortic mesoderm, as well as in the developing epithelial in the liver and thymus [8]. ALCAM expression is developmentally silenced in several adult tissues [13], however, it is re-activated in some mononuclear leukocytes and implicated in monocyte transendothelial migration [15], stabilization of the immunological synapse [3, 16, 17] and in dendritic-cell mediated proliferation of T-lymphocytes [3, 18].

Several studies have reported deregulation of ALCAM expression in multiple cancers. This observation was first made in highly aggressive melanoma cell lines with raised ALCAM mRNA [10]. Subsequently, intense staining of ALCAM protein was reported in invasive melanoma tumors in situ [19], and more recently in primary tumors of the prostate [20, 21], esophagus [22], colon [23] and pancreas [24]. There is an emerging consensus that low level ALCAM mRNA in primary breast cancer tumors is a bad prognostic marker [25-28]. This consensus is supported by observation that patients with the lowest level of ALCAM transcripts developed skeletal metastasis [29], that low ALCAM correlated with an aggressive tumor phenotype and significantly negative correlation between ALCAM expression and tumor diameter and grade [26].

Conversely, high ALCAM expression correlated positively with progesterone and estrogen receptor expression, better response and longer overall survival in patients treated with adjuvant chemotherapy [28, 30]. Most recently, an independent study has shown that low ALCAM mRNA expression in breast cancer tissue was associated with shorter disease free survival and duration of survival (OAS) in a cohort of 481 patients [31].

ALCAM is a junctional adhesion molecule tethered at sites of cell-cell contact in epithelia, mesenchymal, neuronal, and connective tissues. A main function of ALCAM is to mediate appropriate attachment of a cell in its environment. Tumor metastasis involves invasive growth into neighboring tissue, intravasation, survival in circulation, extravasation and colonisation of distant organs [32]. For tumor cells to metastasize, they must alter their adhesion
molecules to detach from the primary tumor mass and then travel to distant sites to establish metastatic lesions [33]. Loss of ALCAM function, due to ALCAM mutant forms, reduced transcript level or low membrane localization, is associated with migration and metastasis in breast cancer.

In this study, we report the cloning and functional characterization of the human ALCAM promoter. A positive regulatory domain with tissue-independent activity was identified in endothelial, epithelial and hematopoietic cells. Two tissue-specific regulatory domains were identified in endothelial and epithelial cells. Basal transcription was dominantly regulated by an Ets binding of cognate motif TCGG in synergy with a downstream Sp1 element.

MATERIALS AND METHODS

Cell culture
Hematopoietic (K562) and epithelial (A549) cell lines were cultured in Iscove’s modified Dulbecco’s medium (IMDM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin sulfate at 37°C under 5% CO₂ atmosphere. Primary rat endothelial cells (PMVECs and PAECs) were isolated as previously described, and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS [15].

Reporter constructs
Human genomic DNA was amplified by PCR using a common reverse primer complimentary to the DNA sequence 60 base pairs (bp) upstream of the translation start site in the ALCAM gene, and various forward primers truncated at 1040, 820, 650, 520, 420 and 220 bp upstream of translation start site (Table 1). PCR products were digested with Kpn I and Sma I, affinity purified, and cloned into a promoter-less luciferase vector (pGL3, Promega, Madison, WI). The ALCAM-luciferase vectors generated (p1000ALCAMLuc, p800ALCAMLuc, p650ALCAMLuc, p500ALCAMLuc, p400ALCAMLuc and p200ALCAMLuc) were verified by sequencing.

Table 1. Sequence of primers and DNA probes.

| ALCAMLuc reporter constructs | Forward                          | Reverse                   |
|------------------------------|----------------------------------|---------------------------|
| -1000                        | 5'-TCTTGGACCCAAGGTTCGCT-3'       | 5'-CCTCCTCCTTCTTGGTG-3'   |
| -800                         | 5'-CAGAAAGTGTTAGTCCAGG-3'       |                           |
| -650                         | 5'-TCAGTCTCGACGCCTCTTA-3'       |                           |
| -500                         | 5'-CGGCCTCCAGTCCCTACT-3'        |                           |
| -400                         | 5'-CCGCCTCTGCGAGTCCTTC-3'       |                           |
| -200                         | 5'-GGTGACCGGGAGGAGGGAGG-3'      |                           |
| -60                          | 5'-CCCTCCTTCTTGGTG-3'           |                           |
Site-directed mutagenesis
2-bp substitutions were generated in individual transcription factor binding sites in the ALCAM promoter in constructs p650ALCAMLuc by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA). The substitutions were -430 GC-box (CCGCC→CCGAA), -550 Sp1 (GGCGG→GGCTT), -580 c-Ets (TGCGTC CCG→TGCGACAGG). Sequence analysis confirmed successful mutations of each cis element.

Reporter gene assays
K562 (5x10^5), A549, PMVECs and PAECs (8x10^4) were seeded in 24-well tissue culture plates. Plasmid DNA of ALCAMLuc reporters (800 ng) and pcDNA3.1/His/LacZ, (100 ng) (Invitrogen) was combined with lipofectamine 2000 and transferred into cells according to the manufacturer’s instructions. For Sp1 trans-activation studies, 50 ng of plasmid DNA expressing Sp1 (pN3Sp1) or an empty vector (pN3) was co-transfected with 500 ng p650ALCAMLuc and pcDNA3.1/His/LacZ (100 ng) plasmid DNA. Twenty-four hours after transfection, cell lysates were prepared and the activities of luciferase (Firefly-Luciferase Reporter Assay System, Promega) and ß-galactosidase (Galacto-Star system, Applied Biosystems) determined using the Veritas Luminometer (Turner Biosystems, Sunnyvale, CA). Luciferase activity was normalized to the activity of ß-galactosidase, and the relative luciferase activity for test constructs calculated by assigning the normalized luciferase activity of the promoter-less pGL3 construct as 1.0. A minimum of three independent experiments were performed for each reporter, each in triplicate.

Electrophoretic mobility shift assay
In vitro protein-DNA interaction was examined using the LightShift Chemiluminescence electrophoretic mobility shift assay (EMSA) kit (Pierce, Rockf ord, IL). ALCAM-specific EMSA DNA probes were synthesized, gel purified and biotin labeled (Integrated DNA Technologies, Coralville, IA). -550 Sp1 probe wt: 5’-GGGAGGAGGCGGGAGAGGA-3’; -550 Sp1 mt: 5’-GGGAGGAGGCTTAGAGAGGA-3’. Nuclear extract (4 µg) was combined with biotin-labeled DNA probes in binding buffer (Pierce, Rockford, IL) containing 50 µg/ml poly(dI-dC) and incubated for 20 min. 50-200-fold molar excess of unlabelled DNA probe was added to the binding reaction in competition experiments. Anti-Sp1 (PEP2) antibodies (2 µg) (Santa Cruz Biotechnology, Santa Cruz, CA) were added and incubated for 24 hours at 4°C. Products of the binding reaction were resolved in a 6% DNA retardation gel (Invitrogen), transferred to a nylon membrane and biotin-labeled complexes detected by chemiluminescence (Fujifilm LAS-1000 imaging system, FujiFilm, Valhalla, NY).
Chromatin immunoprecipitation assay
Protein-DNA cross-linking was performed by fixing 40 million cells with 1% formaldehyde. Nuclei was sonicated on ice in shearing buffer (ChIP-IT, Active Motif, Carlsbad, CA) to obtain chromatin fragments of 1000-200 base pairs, which were pre-cleared with protein G beads (Salmon sperm DNA/Protein G agarose). Pre-cleared chromatin was incubated with anti-sp1 (PEP2) antibody or non-immune IgG. Immune complexes were precipitated with protein G beads, and the elutes were reversed cross-linked in 190 mM NaCl containing RNase A overnight at 65°C, followed by proteinase K digestion at 42°C for 2 hours. DNA was purified and amplified by PCR with specific ALCAM primers 5’-CGCTCATCCGGGCTCCAGGACCG-3’ and 5’-GGCTGCTCTGAGGTAGAGGGACT-3’.

Statistics
Data were analyzed using Graphpad software (Version 5). Student's t-test was used to measure differences in samples of two groups. Probability of less than 0.05 was considered statistically significant. Levels of significance are: p < 0.05 (*), p <0.01 (**) and p < 0.001 (***)

RESULTS AND DISCUSSION
Sequence of the 5' flanking region of the human ALCAM gene
ALCAM expression is widely characterized as tumor promoting in several cancers, it is developmentally regulated in several systems, and it is activated as part of the phenotypic transition by naïve monocytes and subsets of T- and B-lymphocytes into fully differentiated cells [13]. To identify DNA sequences regulating ALCAM gene expression, an approximately 1200 bp Kpn I/Sma I fragment of the ALCAM promoter was amplified by PCR from human genomic DNA. Sequence analysis of multiple clones revealed a complete identity between our clones and several reference sequences in the GenBank database. Fig. 1 shows that the ALCAM promoter contains one copy of a direct repeat sequence, ATTATTATTA, which is present in the Drosophila melanogaster genes encoding transcription factor IIB (TFIIB) and the TATA-box-binding protein (TBP). The 5’-flanking region of ALCAM gene lacks a consensus TATA-box, however the promoter contains several putative regulatory elements, such as AP 2 (-94 and -235), SP1 (-554 and -740), c-Ets (-594), USF (-613) and two aryl hydrocarbon receptor (Ahr/Ar) binding sites (-181 and -700). Moreover, other motifs associated with hemopoietic differentiation were found. One consensus site for NF-E2 is located at -936; three GATA sites at -328, -683 and -848 may direct gene expression during differentiation along the erythroid/megakaryocytic and mast cell lineages [34]; one consensus site for myeloid zinc finger transcription factor MZF1 is located at –582. Also one potential regulatory sequences related to the lymphocytic lineage (LyF-1,-123) was detected. The transcriptional factors in the interval -1000 and -2600 bp of ALCAM gene
promoter were also analyzed using the TRANSFAC 7.0 software. Motifs for NF-KB, NIT2, CdxA, Bcd, Dfd, Gfi-1, Tst-1, IK-1, MyoD, V-myb, MATa1, VBP, Sox-5, SRY, EvI-1, HNF-3b, OCT-X, Pbx-1, NKX-2, Skn-1, CF2-II and Croc were identified. We have previously studied that the NF-KB, at -1140, involved in activating the human ALCAM promoter in melanoma cell lines, but not in breast cancer cell lines [35]. This profile of cis elements is consistent with the fact that ALCAM is expressed in a wide diversity of cell types.

Fig. 1. Nucleotide sequence of a clone of the 5’flanking region of the human ALCAM gene. Putative transcription factor binding sequences are identified and boxed, including AP-2, LyF-1, AhR/Ar, GATA, Sp1, MZF1, c-Ets, MyoD, USF and NF-KB. Sequences for ALCAM reporter construct primers are indicated (underlined).

Tissue-independent activation and tissue-specific silencing of the ALCAM promoter

ALCAM is a cell adhesion molecule expressed by a subset of cells in virtually every system [13], therefore, we begun our functional analysis by examining activity of its promoter in endothelial, epithelial and hematopoietic cells. ALCAM-promoter luciferase reporter gene p200ALCAMLuc had around 10 to 15-fold activity over pGL3 in various cells studied, whereas the relative activity of p400ALCAMLuc ranged 2 to 6-fold over pGL3. The activity of p650ALCAMLuc was significantly higher than that of p200ALCAMLuc. Indeed, it was on average 30-fold higher compared to pGL3 in PMVECs,
PAECs, A549, and K562 (Fig. 2A and B). The different levels of activity from different reporter plasmids suggested that the interval between -400 and -650 in the ALCAM promoter contained ubiquitous cis-acting elements that conferred tissue-independent activation of the ALCAM gene. To test this, the p650ALCAMLuc was transfected into melanoma cell lines, which are of neural crest origin, and remarkably activity of this construct in each cell line was on average 30-fold higher than that of pGL3 (data not shown). This confirmed the idea that DNA sequence localized up to -650 in the ALCAM promoter conferred tissue-independent activation.

Activities of constructs p800ALCAMLuc and p1000ALCAMLuc were tissue-specific. The relative luciferase activity of p800ALCAMLuc was significantly lower than that of p650ALCAMLuc in both types of endothelial cells (Fig. 2A). On the contrary, p800ALCAMLuc showed a similarly high activity as p650ALCAMLuc in K562 and A549 cells. This difference suggested the presence of an endothelial specific-negative regulatory cis element in the interval between -650 and -820 of the ALCAM gene. Activity of construct p1000ALCAMLuc was significantly lower compared to p650ALCAMLuc in K562 and A549 cells (Fig. 2B). Construct p1000ALCAMLuc however produced a similar level of luciferase activity as p650ALCAMLuc in both endothelial cell types (Fig. 2A). Thus, the interval between -820 and -1040 bp of the human ALCAM gene likely contains epithelial cell and hematopoietic cell specific negative regulatory cis elements. Importantly, this result is supported by our previous finding that a negative GATA-1 binding element is in the interval between -820 and -1040 bp of the human ALCAM gene [36]. In said study, we have identified a GATA-1 motif at -850 using the TRANSFAC 7.0 software. This cognate motif inhibited promoter activities in K562 and MEG-01 cells. Mutation of this cis element increased the activity of p1000 ALCAM Luc by 3-fold. We have further confirmed that the ALCAM promoter is occupied by GATA-1 using complement of in vitro and in vivo DNA-protein assays. This analysis is in agreement with other independent studies showing that GATA-1, a transcription factor, is involved in negative regulatory function in hematopoietic cells [37, 38].

Collectively, the data in Fig. 2A and 2B revealed for the first time the presence of positive and negative regulatory domains in the human ALCAM gene. In particular, these data showed the presence of a tissue-independent proximal promoter region involved in basal transcriptional activation of the human ALCAM gene in cells of multiple embryonic origins.

Alteration of adhesion molecule expression is a hallmark of several cancers. In our previous study, we examined the impact of ALCAM on the adhesive behavior of tumor cells in the pulmonary vasculature using the isolated rat lung system [35]. We have found that while MDA-MB-435 cells that do not express ALCAM, can spontaneously metastasize after injection into the mammary fat pad, the MDA-MB-231 cells that express ALCAM cannot metastasize to distant sites from the mammary fat pad. Our results indeed show that loss of ALCAM
expression in MDA-MB-231 and gain of ALCAM expression in MDA-MB-435, switches their adhesive phenotypes in the pulmonary vasculature, a process that influences metastasis to the lung [39, 40].

Fig. 2. A functional Sp1 element in the proximal ALCAM promoter. Schematic diagram of reporter constructs consisting of truncated ALCAM promoter fragments driving expression of pGL3. Histogram shows the relative luciferase activity for each construct in: A – endothelial cells (PMVECs, PAECs) and B – epithelial (A549) and hematopoietic (K562) cells. C – Reporter constructs showing the presence of wild-type (filled circle) or mutant (open circle) cis elements at -430, -550 and -580 in the human ALCAM promoter. Histogram shows the relative luciferase activity for each construct in A549 and K562 cells. Data shown is mean relative luciferase activity for at least three individual experiments, each performed in triplicate.

A canonical Sp1 sequence is essential for optimal ALCAM promoter activity

The above data highlighted an essential role for cis elements located in the interval between -420 and -650 in transcription of the ALCAM gene. Sequence analysis using the TRANSFAC 7.0 software identified multiple putative cis elements in this region, including a canonical Sp1 motif and a c-Ets cis element. In addition, we located a GC-box at -430. Multiple mutations in this GC-box had no impact on promoter activity (Fig. 2C), consistent with results from the p500ALCAMLuc reporter studies (data not shown). On the contrary, activity of p650ALCAMLuc was reduced significantly when the canonical Sp1 element at -550 was mutated (Fig. 2C). As expected no additional reduction in promoter activity was detected by mutating the -430 GC-box element in cis with the -550 Sp1 sequence (Fig. 2C). Mutating the c-Ets element at -580 had no impact on the activity of p650ALCAMLuc, and moreover, this mutation in cis with the -550 Sp1
mutation did not suppress the activity of the ALCAM promoter further (Fig. 2C). We concluded that the canonical Sp1 element at -550 was required for the optimal activity of the proximal human ALCAM promoter.

**Sp1 binds and activates the ALCAM promoter**

The major focus of the current study was to understand how the ALCAM gene is activated. Data from our site-directed mutagenesis experiments suggested Sp1 binding to the canonical element at -550 was essential for optimal activity of the human ALCAM promoter (Fig. 2C). We therefore examined the binding of Sp1 to this cognate motif. Nuclear extracts of K562 and A549 cells formed a single major protein-DNA complex B1, and a faster migrating complex B2 on the -550 ALCAM Sp1 probe (Fig. 3A, lanes 2 and 12). Excess of non-biotinylated probe

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**Fig. 3.** Sp1 binds to the human ALCAM promoter. A – Electrophoretic mobility shift assay showing in lane 1 and 11, wild-type -550 Sp1 probe only. A549 and K562 nuclear extracts form two major complexes B1 and B2 on the wild-type probe (lane 2 and 12). Both complexes are abolished with increasing molar excess of unlabelled probe (lanes 3-5 and 13-15), and attenuated with anti-Sp1 antibody (lane 6 and 16). B1 does not form on a biotin-labeled mutant -550 Sp1 probe (lane 7 and 17). Conversely, unlabeled mutant -550 Sp1 probe does not compete with wild -550 Sp1 probe to form complex B1 (lanes 8-10 and 18-20). B – ChIP strategy used to demonstrate Sp1 occupancy on the endogenous ALCAM promoter. Positions of Sp1 *cis* elements, PCR primers (horizontal arrows) and expected PCR products are indicated. C – Ethidium bromide stained gel showing DNA size marker (lane 1), and 230 bp PCR product (arrow) for input DNA (lane 2), and DNA from chromatin immunoprecipitated with anti-Sp1 antibody (lane 3) or IgG (lane 4).
abolished the assembly of both complexes (lanes 3-5, and 13-15). Anti-Sp1 antibody reduced the intensity of complex B1 but had a marginal effect on complex B2, suggesting that B2 was non-specific. This interpretation was supported by the poor binding of nuclear extracts in experiments performed with biotin-labeled mutant -550 ALCAM Sp1 probe (lane 7 and 17). Moreover, increasing molar excess of unlabeled mutant -550 ALCAM Sp1 probe failed to compete for binding with the wild-type probe (lane 8-10, lane 18-20).

To determine whether Sp1 occupied the endogenous ALCAM promoter, ChIP assays were performed as outlined in Fig. 3B. Briefly, chromatin was immunoprecipitated with anti-Sp1 antibody or non-immune IgG. Fig. 3C showed ethidium-bromide stained PCR products of 230 bp amplified from input (lane 2) and anti-Sp1 chromatin immunoprecipitate (lane 3), using primers flanking the -550 Sp1 motifs. This product was not present in chromatin precipitated with non-immune IgG (lane 4), suggesting that Sp1 occupied the ALCAM promoter. Data from our site-directed mutagenesis (Fig. 2C) and Sp1-DNA binding (Fig. 3) assays supported the idea that Sp1 was involved in transcriptional activation of the human ALCAM gene. To test this idea directly, a DNA construct expressing Sp1 (pN3Sp1) was used in a series of co-trans-activation assays. Co-transfection of 500 ng p650ALCAMLuc with 50 ng of pN3Sp1 increased the activity of the p650ALCAMLuc reporter nearly 3-fold compared to the activity of co-transfection with pN3 plasmid DNA (Fig. 4). In the control experiments, overexpression of Sp1 had no impact on the activity of pGL3, nor did overexpression of the empty vector pN3 influence the activity of p650ALCAMLuc.

![Graph showing luciferase activity](image)

Fig. 4. Sp1 activates the ALCAM promoter. Luciferase activity of pGL3 and p650ALCAMLuc construct in A549 cells transiently over-expressing Sp1 (pN3Sp1) or an empty control expression vector (pN3).

We concluded from these data, and the data from our site directed mutagenesis (Fig. 2C) and Sp1 binding experiments (Fig. 3) that Sp1 activates human ALCAM promoter. The core of this positive regulatory region was mapped to
a site containing a canonical Sp1 binding motif that is flanked upstream by a c-Ets element. Adjacent Ets-like elements influence Sp1 binding to its cognate sequence [41], and several studies have shown synergistic and cooperative transcriptional activation by Sp1 and Ets [41-43]. However, site-directed mutagenesis showed that only the –550 Sp1 element was required for optimum ALCAM promoter activity (Fig. 2C). These findings do not preclude the involvement of other factors in basal activation of the ALCAM promoter. Indeed, putative binding sequences for transcription factors MyoD, USF, MZF1 were identified upstream of the Sp1 element, and some of these elements are known to cooperate with Sp1 to drive transcription of other human genes including N-cadherin [44] and reduced folate carrier B [45]. However, our data clearly establishes a central role for Sp1 in ALCAM promoter activation.

In conclusion, we have characterized the 5′flanking region of the human ALCAM gene and showed that it contains a proximal positive tissue-independent region, two overlapping tissue-specific negative regulatory regions. A proximal 650-bp promoter fragment conferred tissue-independent activation. Basal transcription was dominantly regulated by Sp1 element. Our findings offer new avenues for advancing the understanding of the temporal and disease-specific regulations of the ALCAM gene in other systems.

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