Phorbol Ester and Cyclic AMP-mediated Regulation of the Melanoma-associated Cell Adhesion Molecule MUC18/MCAM

Margaret M. Rummel, Christine Sers, and Judith P. Johnson

Institute for Immunology, Ludwig-Maximilians University, Goethestrasse 31, Munich, 80336 Germany

ABSTRACT

MUC18/MCAM is a melanoma-associated cell adhesion molecule that is also occasionally found on carcinomas and other tumor types. On melanomas, MUC18 expression increases with tumor progression and is found on more than 70% of metastatic lesions. To investigate the regulation of MUC18 expression, cell lines of diverse tissue origin were exposed to cytokines, regulators of intracellular cyclic AMP (cAMP), and to phorbol ester. MUC18 expression could not be induced in negative cell lines and could only be modulated by changes in cAMP levels or by exposure to phorbol ester in positive cells. An increase in intracellular cAMP led to an up-regulation in cell surface MUC18 that was maximal at 48 h. Increased MUC18 mRNA levels were observed as soon as 4 h and were 3-fold higher than in control cells by 48 h. Exposure of the cells to phorbol ester reduced MUC18 surface expression to background levels by 24 h. This down-regulation was associated with decreased mRNA levels that were apparent at 8 h. By 24 h, steady-state levels of MUC18 mRNA had been reduced by 58%. Whereas similar changes in MUC18 surface expression were observed in MUC18-expressing glioma and carcinoma cell lines, melanoma cells were more resistant to the MUC18-modulating effects of cAMP analogues and phorbol ester. These observations suggest that the strong MUC18 expression observed in advanced melanomas may reflect disturbances in the normal regulation of this molecule.

INTRODUCTION

The cell surface glycoprotein MUC18/MCAM was originally identified as a melanoma-associated antigen that increased with tumor progression and metastasis development (1, 2). Whereas MUC18 is only weakly expressed on nevi and thin malignant melanomas, it is a major marker of advanced melanoma and is found on more than 70% of metastases. In nude mice, a correlation has been observed between the presence of MUC18 on human melanoma cells and the ability of these cells to form metastases, suggesting that MUC18 may actually play a role in this process (3). The M, 115,000 MUC18 protein is a novel member of the immunoglobulin superfamily (4) and is most closely related to the chicken neuronal cell adhesion molecules gicerin (5) and SC1/BEN/DM-GRASP (6). Recent studies have shown that MUC18 also functions as a cell adhesion molecule, interacting with an as yet unidentified ligand (7, 8). Although MUC18 expression by tumors is essentially restricted to those observed in melanomas. Here we report that the expression of MUC18 does not appear to be sensitive to regulation by inflammatory cytokines but that it can be up-regulated in cells of diverse tissue origin by exposure to agents that increase intracellular cAMP and down-regulated by exposure to phorbol ester. Although these effects, which are seen at both the protein and mRNA levels, could be readily observed in several different cell types, melanoma cells appeared to be relatively resistant to modulation of MUC18 expression.

MATERIALS AND METHODS

Antibodies, Cells, and Chemicals. The MoAb6 MUC18 (IgG2a) and the MUCB18.3 (IgG1) MoAb, produced against the denatured MUC18 protein, were described by Lehmann et al. (1, 4) The A32 MoAb (IgG1), directed against the extracellular domain of MUC18, was kindly provided by Dr. M. Herlyn (Wistar Institute, Philadelphia, PA; Ref. 4). The mouse myeloma protein UPC10 (IgG2a), was obtained from Sigma Chemical Co. (St. Louis, MO). All cell lines were either purchased from American Type Culture Collection (Rockville, MD), obtained through exchange, or established in our laboratory. Unless otherwise noted, chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture Conditions. All cell lines were cultured in RPMI 1640 supplemented with 5% FCS, 1 mM sodium pyruvate, and antibiotics. PMA was dissolved in acetone (1 mg/ml) and further diluted in culture medium. A 100 mM stock solution of CPT-cAMP was prepared in H2O and stored at -20°C. CPT-cAMP and PMA were freshly diluted for each treatment. For stimulation experiments, cells were seeded into culture flasks and allowed to grow overnight. On the following day, the medium was changed, and the cells were incubated for the given amount of time with PMA or CPT-cAMP at a final concentration of 10 ng/ml and 200 μM, respectively. Control cells were incubated with an equal amount of diluent. For immunofluorescence and RNA preparation, the cells were collected with PBS containing 1 mM EDTA and washed once with PBS.

Immunofluorescence and Flow Cytometry. Indirect immunofluorescence was performed by incubating cells with MoAbs, followed by a FITC-conjugated rabbit antimouse immunoglobulin (Dakopatts, Copenhagen, Denmark). The cells were fixed with 0.1% paraformaldehyde and analyzed using a fluorescence microscope or FACSscan (Becton Dickinson, Mountain View, CA). Corrected mean fluorescence values were calculated by subtracting the mean fluorescence value of the isotype-matched control antibody (UPC10) from the mean fluorescence value of the specific antibody (MUC18). ΔMC represent the differences between the corrected mean channel values of the treated and untreated cells.

Immunoprecipitation and Western Blotting. Cells (6-7 x 10⁶) per sample were lysed in PBS containing 1% NP40 (Calbiochem, San Diego, CA), 1 mM benzamidine hydrochloride, and 1 mM phenylmethylsulfonyl fluoride. Fifty ml of cell culture supernatant were collected per sample, and both lysates and supernatants were precleared by overnight precipitation at 4°C with horse serum saturated Sepharose. After preclearing, the samples were exposed to MUC18 MoAb bound to Sepharose protein A beads overnight at 4°C. The bound material was eluted from the washed immunosorbents by heating the samples for 5 min at 95°C in SDS-PAGE sample buffer. Twenty-five μl (equivalent to 3 x 10⁶ cells or 50 ml culture supernatant) were loaded per lane and separated under reducing conditions together with prestained molecular weight markers.

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2To whom requests for reprints should be addressed. Phone: 49-89-5996-660; Fax: 49-89-5160-2353; E-mail: johnson@ifi.med.uni-muenchen.de.

3The MUC18 gene has been given the official name of MCAM by the Genome Data Base.

4Margaret M. Rummel and Judith P. Johnson, unpublished observations.

5Base 5 Margaret M. Rummel and Judith P. Johnson, unpublished observations.

6The abbreviations used are: MoAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; CPT-cAMP, 8-(4-chlorophenylthio)-cAMP; ΔMC, delta mean channel values; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FACS, fluorescence-activated cell sorter; IL, interleukin.
weight markers (M₁, 14,300–200,000; Rainbow Markers, Amersham, United Kingdom) on a 7.5% SDS-PAGE gel (11).

The gels were electrophoretically blotted to nitrocellulose filters (BA85; Schleicher and Schuell, Dassel, Germany), blocked overnight in PBS containing 0.1% gelatin, and incubated with either MoAb MUCBA18.3 or MoAb A32. The blots were washed in PBS/0.05% Tween-20, incubated with rabbit antimouse peroxidase-conjugated antiserum (P161; Dakopatts), and washed in PBS/Tween. The bound antibody was visualized by using a substrate solution containing 1.26 mm o-dianisidine hydrochloride, 15.3 mm sodium nitroprussic acid, and 0.007% H₂O₂.

Northern Blotting. Total RNA was prepared from control and treated cell lines according to the method of Okayama et al. (12). Twenty μg of RNA were loaded per lane, separated on a 1.2% agarose/formaldehyde gel, and transferred to a Hybond N (Amersham) nylon membrane. The full-length MUC18 cDNA clone, Drop4.7, was labeled with [α-32P]dATP (Amersham) by random priming (13). Hybridization was performed overnight at 65°C in 6× SSC (1× SSC, 0.15 n NaCl, 0.015 m sodium citrate, pH 7), 5× Denhardt’s solution, and 0.5% SDS. The blots were washed at 65°C in 3× SSC/0.1% SDS, 1× SSC/0.1% SDS, 0.3× SSC/0.1% SDS, 0.1× SSC/0.1% SDS, and exposed to X-ray film.

Filters were rehybridized with the oligonucleotide complementary to the first exon of human GAPDH (14). MUC18- and GAPDH-specific bands on the autoradiograms were analyzed for relative MUC18 expression using an ELScript 400 densitometer (Hirschmann, FRG). The densitometric value for the signal from untreated cells was set at 1.0, and the relative MUC18 expression for the corresponding treated signal was calculated as a percentage of the control value.

RESULTS

Modulation of MUC18 Expression in Cell Lines. Seventy-eight cell lines of diverse tissue origin were examined for the expression of MUC18 surface protein using immunofluorescence. Expression was found to be characteristic for cells of neuroectodermal origin and was observed in melanomas (30 of 34 examined), gliomas (7 of 10), and neuroblastomas (2 of 3). Expression was not observed on T-cell lymphomas (2/2) or B-lymphoblastoid cell lines (3/3) but was seen on 3 of 24 carcinomas tested: SW480 (colon), SKBR3 (breast), and HeLa (cervix). MUC18 was also detected on a leiomyosarcoma (SKLMS-1) and on a SV40 transformed fibroblast cell line, SV40-HF.

To determine if it was possible to modulate MUC18 expression, the majority of these cell lines were exposed to various cytokines, PMA, and forskolin, an activator of adenylyl cyclase that leads to increased cAMP levels. None of these agents was able to induce MUC18 expression in any negative cell line. Exposure to IL-1, IL-2, IL-4, and IL-6, tumor necrosis factor-α, or IFN-γ had no influence on MUC18 expression in any of the seventy-eight cell lines examined. Conversely, exposure to PMA or forskolin led to clear changes in MUC18 surface expression in the gliomas, SKLMS-1, SV40-HF, and the three MUC18-positive carcinoma cell lines. To investigate the regulation of MUC18 expression by PMA and cAMP in greater detail, the glioma cell line LN215, which was representative of the responding cells, was chosen for additional study.

Increased cAMP Levels Up-Regulate MUC18 Expression. Exposure of MUC18-positive carcinoma, leiomyosarcoma, glioma, and transformed fibroblast cell lines to forskolin led to an increase in MUC18 surface expression (data not shown). Similar results were obtained with isobutylmethylxanthene (an inhibitor of phosphodiesterase) and with the stable cAMP analogue, CPT-cAMP. As examined in detail in the glioma cell line LN215, treatment of cells with CPT-cAMP led to an increase in MUC18 surface expression that was maximal after 48 h. This up-regulation is reflected in the corresponding FACS histogram shifts and corrected mean channel values (Fig. 1, A and B). A 130-channel increase was recorded for the treated cells compared to the untreated population (Fig. 1, A and B). During this time, no morphological changes in the cells were observed.

Northern blot analyses were performed to determine if CPT-cAMP affected the level of MUC18 mRNA in LN215 cells. Total cellular RNA was isolated from treated and untreated cells and hybridized with the full-length MUC18 cDNA clone, Drop4.7. To correct for sample loading errors, the blot was also hybridized to a probe for the enzyme GAPDH, and the relative amounts of MUC18 mRNA were determined by densitometric evaluation. A nearly 3-fold increase in the relative levels of the 3.3-kb MUC18 mRNA was seen after 48-h treatment with CPT-cAMP (Fig. 2A). A kinetic study profiling the effects of CPT-cAMP indicated that an increase in MUC18 mRNA could be seen very early (Fig. 3). A 2.2-fold increase in the level of MUC18 mRNA was already observed at 4 h, and levels rose fairly steadily over 60 h of treatment, reaching a nearly 6-fold increase over the untreated control at the 60-h time point.

Exposure to Phorbol Ester Leads to a Down-Regulation in MUC18 Expression. Treatment of MUC18-positive glioma, carcinoma, leiomyosarcoma, and transformed fibroblast cell lines with PMA led to a loss of MUC18 surface expression that was most apparent after 24 h. The effect of PMA exposure on MUC18 antigen levels on LN215 cells after 24 h was quantitated through FACS analyses (Fig. 1, C and D). The histogram shifts and corrected mean channel values show that the MUC18 expression is suppressed to below background levels after PMA treatment (Fig. 1, C and D). Microscopic evaluation of the cells revealed no obvious morphological changes following this treatment. MUC18 down-regulation did not require the continual presence of PMA and was also observed after short exposures of 15, 30, and 60 min. However, in all cases, the maximal decline in MUC18 surface levels was observed at 24 h following exposure to PMA. The observed loss of MUC18 cell surface expression could be due to PMA-induced shedding or internalization of the molecule, as has been reported for other cell surface molecules (15, 16). To investigate this, the supernatant of PMA-stimulated cells was examined for the presence of soluble MUC18 protein. Immunoprecipitation of stimulated (24 h; PMA) and unstimulated cells was performed to determine if CPT-cAMP affected the level of MUC18 mRNA in LN215 cells. Total cellular RNA was isolated from treated and untreated cells and hybridized with the full-length MUC18 cDNA clone, Drop4.7. To correct for sample loading errors, the blot was also hybridized to a probe for the enzyme GAPDH, and the relative amounts of MUC18 mRNA were determined by densitometric evaluation. A nearly 3-fold increase in the relative levels of the 3.3-kb MUC18 mRNA was seen after 48-h treatment with CPT-cAMP (Fig. 2A). A kinetic study profiling the effects of CPT-cAMP indicated that an increase in MUC18 mRNA could be seen very early (Fig. 3). A 2.2-fold increase in the level of MUC18 mRNA was already observed at 4 h, and levels rose fairly steadily over 60 h of treatment, reaching a nearly 6-fold increase over the untreated control at the 60-h time point.

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REGULATION OF MUC18/MCAM EXPRESSION

Fig. 2. A. MUC18 mRNA expression in LN215 cells treated with CPT-cAMP. Total RNA from treated and untreated cells (20 μg/lane) was hybridized to the full-length MUC18 cDNA. The MUC18 3.3-kb message is indicated on the left, together with the internal control, GAPDH. Lane 1, 48 h (−) CPT-cAMP; Lane 2, 48 h (+) CPT-cAMP. The bar graph shows the relative MUC18 levels as determined by densitometric analysis. Lane 1, 48 h (−) CPT-cAMP; Lane 2, 48 h (+) CPT-cAMP. B. MUC18 mRNA expression in LN215 cells treated with PMA. Lane 1, 24 h (−) PMA; Lane 2, 24 h (+) PMA. The bar graph shows the relative MUC18 levels as determined by densitometric analysis. Lane 1, 24 h (−) PMA; Lane 2, 24 h (+) PMA. The blots pictured are representative of three experiments with similar results.

Fig. 3. Kinetics of MUC18 mRNA expression in LN215 cells treated with CPT-cAMP. Total RNA (20 μg/lane) from untreated (U) or treated (T) LN215 cells was hybridized to the full-length MUC18 cDNA. The Northern blot in the lower half of the figure shows the MUC18 3.3-kb message and the corresponding GAPDH signal for treated and untreated cells at the various time points. The bar graph shows the relative MUC18 levels as determined by densitometric analysis. The data are representative of two experiments with similar results.

Fig. 4. Western blot analysis of MUC18 protein expression in cells and culture supernatant following PMA treatment. MoAb MUC18 immunoprecipitates were separated on an SDS-PAGE gel and blotted on to nitrocellulose filters in the following order: Lane 1, negative control, SBC12 (a MUC18-negative cell line) cell culture supernatant; Lane 2, untreated LN215 cell lysate; Lane 3, untreated LN215 cell culture supernatant; Lane 4, M, 14,300–200,000 prestained molecular weight markers; Lane 5, 24 h PMA-treated LN215 cell lysate; Lane 6, 24 h PMA-treated LN215 cell culture supernatant; Lane 7, positive control, MelJuSo (a MUC18-positive cell line) cell lysate. The filter was stained with MoAb A32. Arrow, the position of the MUC18 glycoprotein with an apparent molecular weight of M, 113,000 was detected in the untreated LN215 cell lysate (Fig. 4, Lane 2) and in the control MelJuSo lysate (Fig. 4, Lane 7). In contrast, MUC18 glycoprotein could not be detected in the PMA-treated LN215 cell lysate (Fig. 4, Lane 5), untreated LN215 supernatant, or PMA-treated LN215 supernatant (Fig. 4, Lanes 3 and 6, respectively). This finding suggests that the observed loss of MUC18 surface expression is not occurring through shedding or proteolytic cleavage from the cell surface.

The effect of PMA on relative MUC18 mRNA levels in LN215 cells was examined by hybridizing total RNA samples from PMA-treated and untreated cells with the Drop4.7 clone. A 58% decrease in the relative amount of MUC18 mRNA was seen after 24 h incubation with PMA (Fig. 2B). Kinetic studies showed that a 20% reduction in MUC18 mRNA levels could be observed as soon as 8 h after PMA treatment and reached 60% by 18 h (Fig. 5). These findings indicate that the suppression of MUC18 cell surface expression by PMA is occurring at the level of the mRNA.

Decreased Sensitivity of Melanoma Cell Lines to MUC18 Modulation by cAMP and Phorbol Ester. In contrast to MUC18-positive glioma and carcinoma cells, preliminary observations indicated that melanoma cells displayed a reduced sensitivity to the MUC18-modulating effects of cAMP and PMA. To examine this more closely, melanoma cell lines with MUC18 expression levels similar to LN215 cells were selected for further study (Figs. 1, 6, and 8).

The MUC18 positive melanoma cell lines A375, Mel Ei, and
Whereas the CPT-cAMP-treated LN215 cells (AMC 130) showed an increase in relative MUC18 mRNA levels observed in LN215 cells after treatment with CPT-cAMP (Fig. 2A).

A375, Mel Ei, and SkMel 23 cells were incubated with PMA for 24 h and then analyzed for changes in MUC18 surface expression. Small but apparent negative shifts in fluorescence intensity relative to the untreated cells were seen in all FACS histograms (Fig. 8). ΔMC values for A375, Mel Ei, and SkMel 23 cells (18, 53, and 32, respectively) reflect small decreases when compared with LN215 cells (ΔMC 209; Fig. 1, C and D). Northern analyses revealed no decrease in the relative MUC18 mRNA amounts in A375 and SkMel 23 cells and, in fact, a slight (37%) increase was observed in Mel Ei cells (Fig. 9). This is in contrast to the 58% decrease in relative MUC18 mRNA levels seen in similarly treated LN215 cells (Fig. 2B).

**DISCUSSION**

The melanoma-associated cell adhesion molecule MUC18 increases in expression during the growth of primary tumors, becoming an important marker of metastatic disease (17). Southern blot analysis and sequencing of MUC18 cDNA from melanoma cells indicate that the increased expression on these tumors is not due to translocation, amplification, or mutation of the gene (3, 6) and suggest that MUC18 expression may be induced by environmental signals. The studies reported here indicate that cytokines likely to be produced by infiltrating leukocytes or neighboring keratinocytes have no apparent influence on MUC18 expression. In a variety of cell types, however, MUC18 expression can be enhanced by increased intracellular cAMP and down-regulated by the protein kinase C agonist PMA.

As analyzed in detail in the glioma cell line LN215, MUC18 mRNA levels show an increase following treatment with CPT-cAMP, a stable analogue of cAMP. This initial increase was observed already at 4 h and continued through at least 60 h. Sequence analysis of the MUC18 promoter has revealed the presence of two putative cAMP-responsive elements, TGACGTCA (3). One is located 32 bp upstream of the transcriptional start, and the second is in the first intron. The early increase in MUC18 expression observed in response to raised cAMP levels suggests increased transcription via one of these cAMP-responsive element sites. The appearance of a further increase after 48 h may indicate the action of a molecule that is itself first induced by cAMP.

In contrast to the effects of cAMP, the exposure of cells to PMA led to a decrease or complete loss of MUC18 cell surface expression.
Fig. 7. MUC18 mRNA levels in melanoma cells treated with CPT-cAMP. Total RNA (20 μg/lane) from untreated (U) or treated (T) A375, Mel Ei, or SkMel 23 cells were hybridized to the full-length MUC18 cDNA. The Northern blot shows the MUC18 3.3-kb message and the corresponding GAPDH signal for the given time points. Lane 1, A375 U; Lane 2, A375 T; Lane 3, Mel Ei U; Lane 4, Mel Ei T; Lane 5, Sk Mel 23 U; Lane 6, Sk Mel 23 T. The bar graph shows the relative MUC18 levels as determined by densitometric analysis. The data are representative of two experiments with similar results.

PMA-induced internalization (relocalization), shedding, or proteolytic cleavage have been demonstrated for other cell surface molecules (15, 16) and pose possible explanations for the loss of MUC18 expression. The absence of detectable MUC18 antigen, both in the supernatant and lysate of the treated cells, argues against these mechanisms, although extensive proteolysis of shed protein would have been undetectable. Analysis of the mRNA kinetic profile in PMA-treated glioma cells indicates that the loss of MUC18 expression is due to changes in mRNA levels. Whether this reduction in MUC18 mRNA is due to increased degradation or to a reduction in transcription remains to be determined. A classical TPA responsive element (18) has not been identified in either the MUC18 promoter or the first intron. However, as phorbol ester may induce or activate other transcription-regulating factors (19, 20), an effect on transcription itself cannot be ruled out.

Whereas PMA and cAMP had similar effects on MUC18 expression in cells derived from a variety of tissues, the melanomas examined appeared to be more resistant to these regulatory influences. Although slight changes in the MUC18 surface expression were seen in all PMA-treated cells and in one CPT-cAMP treated cell, this was not accompanied by corresponding changes at the mRNA level. Because of the small number of MUC18-positive cell lines examined and the lack of information on MUC18 expression on gliomas in vivo, it is not clear if these differences are related to tumor type or tumor stage. Nevertheless, our findings may indicate that the high expression of MUC18 observed on advanced melanomas is due to a disturbance in its normal regulation. Whether this reflects a more general alteration in particular signaling pathways in melanomas remains unclear. Melanoma cells express both protein kinase A regulatory and catalytic subunits and the α isoform of protein kinase C (21). However, melanoma cells have been reported to be unresponsive to the growth and morphology altering effects of increased cAMP levels (22) and are resistant to keratinocyte-induced changes in antigen expression (23).

Although these results indicate that MUC18 expression can be modulated by a protein kinase C agonist and by changes in cAMP levels, the signals that activate these pathways in vivo have not yet been identified. Recent studies in two different systems suggest that cell adhesion-mediated, or juxtacrine, signaling (24) may regulate MUC18 expression. In contrast to the situation in vivo, epidermal melanocytes cultured in vitro express MUC18 on their cell surface. Shih et al. (23) observed that this expression was lost when the melanocytes were cultured for several days together with keratinocytes. This effect required cell-cell contact, suggesting that certain intercellular interactions may mimic PMA and reduce MUC18 expression. Ectopically expression of the cell adhesion molecule, carcinoembryonic antigen, by melanoma cells can lead to an increase or decrease in homotypic cell adhesion, depending on the cell line (25). In cell lines where carcinoembryonic antigen expression leads to a decrease in homotypic adhesion, a specific up-regulation of MUC18

Fig. 8. FACS analysis of MUC18 expression on PMA-treated melanoma cells. Immunofluorescence staining was performed on control and PMA-treated A375, Mel Ei, and SkMel 23 cells. Each panel shows the log fluorescence intensity (X axis) versus cell number (Y axis). Unshaded histograms, the isotype control UPC10; shaded histograms, MUC18 fluorescence. Corrected mean channel values (in the upper-right corner of each panel) were calculated as described in "Materials and Methods." Each histogram is derived from 5000 events and is representative of three experiments with similar results.

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The bar graph shows the relative MUC18 levels as determined by GAPDH correlation between the reduction in cell-cell interactions and an increase in MUC18 expression. These observations raise the possibility that the up-regulation of MUC18 expression observed in vitro as melanomas increase in vertical thickness is the direct result of the loss of normal adhesive interactions with keratinocytes or cellular elements in the dermis. As melanomas progress and develop metastatic lesions, genetic alterations and changes in gene expression become possible to devise strategies to down-regulate expression of this lineage by two novel antigens, a glycoprotein with a molecular weight of 113,000 and a protein with a molecular weight of 76,000. Cancer Res., 47: 841–845, 1987.


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