Lineage-specific Signaling in Melanocytes

c-Kit STIMULATION RECRUITS p300/CBP TO MICROPHTHALMIA

During melanocyte development, the cytokine Steel factor activates its receptor c-Kit, initiating a signal transduction cascade, which is vital for lineage determination via unknown downstream nuclear targets. c-Kit has recently been found to trigger mitogen-activated protein kinase-mediated phosphorylation of Microphthalmia (Mi), a lineage-restricted transcription factor, which, like Steel factor and c-Kit, is essential for melanocyte development. This cascade results in increased Mi-dependent transcriptional reporter activity. Here we examine the mechanism by which Mi is activated by this pathway. Phosphorylation does not significantly alter Mi’s nuclear localization, DNA binding, or dimerization. However, the transcriptional coactivator p300/CBP selectively associates with mitogen-activated protein kinase-phosphorylated Mi, even under conditions in which non-MAPK phospho-Mi is more abundant. Moreover, p300/CBP coactivates Mi transcriptional activity in a manner dependent upon this phosphorylation. Mi thus joins CREB as a transcription factor whose signal-responsive phosphorylation regulates coactivator recruitment, in this case modulating lineage development in melanocytes.

Mutations in the genes that encode the receptor tyrosine kinase c-Kit (1), its ligand Steel factor (SI, mast/stem cell growth factor) (2, 3), and the basic/helix-loop-helix/leucine zipper transcription factor Microphthalmia (Mi) (4) all produce severe melanocyte deficiency in mouse. This striking phenotypic overlap has led to the suggestion that Sl, c-Kit, and Mi function in a common growth/differentiation pathway (5, 6). In human, c-Kit mutation is associated with patchy depigmentation called piebaldism (7), and Mi mutation causes Waardenburg Syndrome type II marked by depigmentation and deafness because of pigment cell deficiency in the skin and inner ear (8). Mi has been shown to modulate c-Kit expression levels in mast cells (6). Recent studies have also biochemically linked Mi to the c-Kit signaling pathway (9). In response to SI stimulation, c-Kit signaling activates MAP kinase (MAPK/Erk), which directly phosphorylates Mi at serine 73 (Ser-73), based on two-dimensional tryptic analyses of in vitro and in vivo phosphorylated Mi protein (9). This phosphorylation is associated with increased expression of a reporter driven by the tyrosine-signal transduction gene promoter.

Mi is a highly tissue-restricted dimeric transcription factor, which recognizes “E box”-containing promoter/enhancer elements (4, 10–12). Among its transcriptional targets are the melanocyte-specific tyrosine-metabolizing enzyme genes involved in melanin biosynthesis. In addition to the phenotypic similarity of c-Kit, Sl, and Mi mutant mice, recent clinical use of recombinant SI in humans has demonstrated both proliferative and pigmentation responses in melanocytes near the injection site (13). These observations suggest that c-Kit signaling proceeds through regulated activation of Microphthalmia, which in turn initiates the pigmentation program and activates unknown genes conferring survival during development.

Phosphorylation can regulate transcription factor activity by a variety of mechanisms. These include altered nuclear localization as with STAT (14) and NFAT factors (15), altered dimerization specificity as with MyoD (16), altered DNA binding as with Max (17), or altered protein stability as with c-Jun (18). To determine the mechanism whereby phosphorylation of Mi alters its transactivation potential, several strategies were employed. Immunostaining and DNA binding assays showed no phospho-regulated difference in subcellular localization or altered DNA binding. Recently, Mi was found to employ the transcriptional coactivator p300/CBP (19). Here we further show that p300/CBP selectively associates with and activates MAPK-phosphorylated Mi in both binding and functional assays in vivo. This mechanism explains the recently reported c-Kit-dependent up-regulation of Mi function (9), corroborated here in functional assays measuring Mi transactivation in vivo. More generally, these observations link extracellular cytokine stimulation to regulated transcriptional programs on chromatin in melanocytes.

MATERIALS AND METHODS

Immunofluorescence—501mel cells, plated on 18-mm glass coverslips, were treated with or without 1 μg/ml Steel factor (Sl) (R & D Systems) for 15 min. The cells were fixed with 3% formaldehyde and incubated with both mouse anti-Mi (DS) and rabbit anti-Erk1/2 (NEB) followed by fluorescein isothiocyanate-conjugated goat anti-mouse and Cy3-conjugated goat anti-rabbit (Jackson Immunological). Nuclei were stained with 10 ng/ml DAPI (Sigma). All incubations were followed by three washes with 0.1% Triton X-100 in phosphate-buffered saline.

Transfection Studies—Transfections into BHK cells were performed in 24-well plates using lipofectAMINE according to manufacturer’s instructions (Life Technologies, Inc.). For each well 0.7 μg of total DNA in 30 μl of serum-free Dulbecco’s modified Eagle’s medium was mixed with an equal volume of 5% lipofectAMINE in Dulbecco’s modified Eagle’s medium. Two days later, cells were harvested and luciferase assays performed using dual luciferase reagents (Promega). Luciferase values were normalized for transfection efficiency using cotransfected sea pansy luciferase plasmid (Promega).

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c-Kit Signaling Recruits p300/CBP to Mi

RESULTS AND DISCUSSION

When c-Kit-expressing melanoma cells are treated with SI factor, MAPK is rapidly activated, leading to the S73 phosphorylation and a mobility shift in Mi that is blocked by inhibition of MAPK (Fig. 1A). The consequences of this modification were examined with respect to Mi's subcellular localization and DNA binding activity. Immunofluorescence demonstrated that although SI activation stimulates distinct MAPK translocation from cytoplasm to nucleus, SI stimulation does not alter endogenous Mi's nuclear localization (Fig. 1B). Electrophoretic mobility shift analysis showed no significant difference in Mi's DNA binding propensity in nuclear extracts from resting or SI-stimulated cells (Fig. 1C, lanes 3 and 4). Because a variety of ubiquitous factors are capable of binding the same DNA sequences as Mi, an antibody-mediated supershift was employed to identify Mi-specific binding. The absence of a significant change in Mi's DNA binding activity suggests that endogenous protein dimerization is also minimally affected by c-Kit-induced phosphorylation. This signaling pathway stands in contrast to other pathway inducers such as the melanocortins, which may specifically alter Mi DNA binding levels (data not shown and [22]). Because Mi phosphorylation at S73 does not significantly affect nuclear localization or the assembly of

DNA-binding dimers in vivo, it was possible that phosphorylation more directly influenced transactivation potential, perhaps through interactions with transcriptional cofactor(s).

The adenovirus E1A protein was previously shown to produce hypopigmentation in melanocytes as a consequence of down-regulating genes encoding pigment synthetic enzymes, such as tyrosinase and TRP-1 [23, 24]. In cotransfection studies, E1A profoundly repressed Mi-dependent transactivation of the tyrosinase promoter (Fig. 2A, lanes 3 and 4). The Mi-dependent increase in luciferase activity was blocked at basal levels by cotransfection with E1A (Fig. 2A, lanes 1-4). E1A is known to bind and inactivate both p300 and the retinoblastoma protein, Rb [25]. E1A's ability to inhibit Mi-dependent transactivation correlated with the ability of mutant E1A to bind and sequester p300 (Fig. 2A, lanes 4-7). This dependence is further supported by the observation that E1A repression can be rescued by overexpression of p300 in a dose-dependent manner (Fig. 2B, lanes 6-8). As with many p300/CBP coactivator systems, transfected CBP has been shown to superactivate Mi-dependent transcription as well (Ref. 19 and see below).

Direct evidence for physical interaction of Mi with p300 was assessed by immunoprecipitation/Western analysis of the endogenous proteins in melanoma cells (Fig. 3, A and B). Antibodies raised against p300 monoclonal, but not isotype-matched control antibody, immunoprecipitated endogenous Mi in human melanoma (501mel) cell extracts (20). For cells stimulated by Steel factor, Mi migrates as a single species and approximately 10% of the input Mi protein is associated with p300 (Fig. 3A). Mi extracted from cells grown in the absence of exogenous Steel factor mi-
Mi to the Gal4 DNA binding domain (1–147). (data not shown). Chimeras were generated by fusion of residues 1–147 to the Gal4 DNA binding domain alone (DBD) or this domain fused to the N-terminal 147 residues of Mi containing serine (Ser) or alanine (Ala) at position 73. These transfection conditions were found to activate endogenous MAPK (9), these data demonstrate that endogenous Mi and p300 are present within a complex and that S73 phosphorylation selectively enhances the Mi-p300 interaction.

To test the functional consequences of this phosphorylation-dependent interaction, p300-mediated superactivation was examined in cells cotransfected with chimeras containing the Gal4 DNA binding domain linked to an N-terminal fragment of Mi which includes S73 as well as the adjacent transactivation domain, but which excludes a C-terminal transactivation domain, but which excludes a C-terminal transactivation domain. Engagement of the coactivator p300. These studies demonstrate that Sl/c-Kit activation triggers a signaling cascade, which leads to a modification of the Mi protein p300 was initially identified by its ability to bind E1A in E1A-transformed cells and, when cloned, was found to be related to the CREB-binding protein, CBP (26, 27). Both p300 and CBP are transcriptional coactivators, which may contact RNA polymerase holoenzyme (28) as well as catalyze histone acetylation by either their own intrinsic activity (29, 30) or by recruitment of other histone acetyltransferases (31). Because Mi can interact with p300/CBP (Figs. 2 and 3 and Ref. 19) in a phospho-specific fashion (Figs. 2 and 3), Mi immunoprecipitates were also tested for the presence of HAT enzymatic activity. Extracts from either melanoma cells or Mi-transfected BHK cells were immunoprecipitated with either anti-p300, anti-Mi, or control antibodies, and the resulting immunoprecipitates were assayed for HAT activity in vitro (Fig. 4A). Compared with total anti-p300 immunoprecipitable HAT activity, immunoprecipitates of endogenous Mi contained significant HAT activity (~16% of total) (Fig. 4A, lanes 1 and 2). Reproducible Mi-associated HAT activity was also observed in Mi-transfected BHK cells, albeit with more modest induction over background. This is presumably related to cell type differences and transfection efficiency (Fig. 4A, lanes 3-6). Consistent with p300’s widespread role as a transcriptional coactivator, the Mi-associated HAT activity represented only a fraction of the total activity measured in direct p300 immunoprecipitates (Fig. 4A).

These studies demonstrate that Sl/c-Kit activation triggers a signaling cascade, which leads to a modification of the Mi transcription factor thereby selectively enhancing recruitment of the coactivator p300. Engagement of p300 may induce transcriptional activity by several mechanisms, including cooperativity with promoter-bound factors (scaffolding-like effect), enhanced interactions with RNA polymerase holoenzyme, and chromatin remodeling via alterations in histone acetylation. Future studies, which examine chromatin-based functional assays, may demonstrate larger differences than those seen here, because it is uncertain to what degree plasmid reporters recapitulate p300’s in vivo functions. HAT assays of Mi immunoprecipitates have also shown reproducibly enhanced activity in response to c-Kit stimulation (30–100% increase (data not shown)). The magnitude of this difference appears to reflect the presence of significant phospho-Mi in nonstimulated cells (Fig. 4A and Ref. 9). Although the mechanisms employed by p300/CBP to transmit Mi’s activation signal remain to be fully elucidated, these observations link critical environmental signals to specific transcriptional programs central to lineage survival in development and may be studied using in vitro melanocyte differentiation systems (32).

The exact means by which Mi phosphorylation biochemically
stabilizes p300 binding is not yet understood. Separate studies have suggested that p300/CBP can interact separately with a short transactivation motif approximately 40 residues downstream of S73 (data not shown and Ref. 19). In the intact protein, phosphorylation could alter the affinity or accessibility (or both) of the activation domain for p300/CBP. Because this activation domain encodes a putative acidic amphipathic α helix (33), repulsion between S73-phosphate and the acidic activation domain could trigger a folding transition, thereby enhancing accessibility of the activation domain to p300/CBP.

The striking phenotypic similarity of mice carrying mutations in c-Kit, Sl, and Mi is consistent with evidence that these factors lie within a common signal transduction cascade critical for lineage survival. Genetic studies in mice have repeatedly shown that cell type-specific phenotypes can indeed link loci that encode proteins that interact at the biochemical level. For example, mutations in the tyrosine receptor kinase c-Kit can compensate for mutant alleles at the motheaten locus, which encodes a tyrosine phosphatase (34, 35). Humans harboring heterozygous germ line mutations of the CBP gene are afflicted with Rubinstein-Taybi syndrome manifested by facial abnormalities, broad thumbs, and mental retardation (36). There are reports of affected individuals with Rubinstein-Taybi syndrome displaying piebaldism (37) and ocular defects (38), which are potentially consistent with p300/CBP’s role in modulation of transcription factor whose interaction with p300/CBP occurs in a signal/macrophase (42) and keratinocyte (43) differentiation, though via different regulatory mechanisms.

Mi represents a novel example of a tissue-specific transcription factor whose interaction with p300/CBP occurs in a signal/phosphorylation-dependent manner. The only other known phosphorylation-specific CBP/p300 interaction occurs with CREB (44–46), albeit not in a fashion thought to influence lineage specific development/survival. c-Jun interacts with CBP in a manner requiring a critical serine, but apparently not involving phosphorylation (47). The Sl/c-Kit/Mi regulatory pathway provides a unique example wherein general signaling (MAPK) and coactivation (p300/CBP) machineries convey highly restricted signals, based on tissue-specific cytokine receptor and transcription factor targets in development.

Acknowledgments—We are grateful to J. DeCaprio and B. Moran for E1A plasmids; N. Moghal and B. G. Neel for Gal4 plasmid; and to the members of the Fisher laboratory, especially S. Rowan for advice and assistance. We also thank D. M. Livingston for advice. 501mel cells were generously provided by R. Halaban.

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