Binding of α-melanocyte-stimulating hormone to its G-protein-coupled receptor on B-lymphocytes activates the Jak/STAT pathway

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α-Melanocyte-stimulating hormone (α-MSH) is a 13-amino-acid peptide with a variety of physiological effects, including the stimulation of melanocyte proliferation and melanogenesis, temperature control, control of prolactin release and the modulation of cytokine action in the immune system. There are five known subtypes of G-protein-coupled receptors, which bind with different affinities to α-MSH. This paper provides evidence that Ba/F3 pro-B-lymphocyte cells express the gene for the melanocortin 5 (MC5) receptor and specifically bind α-MSH. Western-blot analysis reveals that α-MSH binding stimulates Janus kinase 2 (JAK2) and signal transducers and activators of transcription (STAT1) tyrosine phosphorylation in both Ba/F3 cells and human cultured IM-9 lymphocytes. α-MSH is further revealed to activate JAK2 in mouse L-cells stably expressing the human MC5 receptor. Finally, α-MSH binding is shown to result in an enhancement of cellular proliferation. These findings identify a new protein tyrosine kinase pathway in the action of α-MSH, and suggest that α-MSH plays an important role in B-lymphocyte function via the activation of the same intracellular phosphorylation pathway used by cytokines and growth factors.

INTRODUCTION

Pro-opiomelanocortin (POMC) is a peptide hormone precursor which is expressed primarily in the pituitary gland, as well as in the brain and periphery [1,2]. POMC is processed post-translationally to give rise to a number of biologically active hormones known as melanocortins (MCs), which have very diverse physiological functions [3]. The family of MCs includes adrenocorticotropic hormone (ACTH), α-, β- and γ-melanocyte-stimulating hormone (α-, β- and γ-MSH respectively) and β-endorphin. α-MSH, a 13-amino-acid neuroendocrine hormone, was first described as a stimulator of melanocyte growth and pigment production, and is now known to affect fever, inflammation and the immune response [3–5].

α-MSH, and other MC peptides, are known to have specific effects on immune system cells [3]. For example, α-MSH: (i) inhibits interleukin (IL)-1-induced cellular proliferation of mouse thymocytes [4]; (ii) inhibits the chemotaxis of mouse neutrophils towards cytokine chemoattractants [6]; and (iii) affects mouse T- and B-lymphocyte, and natural killer cell, toxicity in response to shock [7], whereas ACTH modulates human B-cell growth and differentiation [8]. Thus MCs have broad and varied effects on a variety of immune system cells. No second messenger has been identified in immune cells following stimulation with MCs.

There have been five subtypes of MC receptors cloned to date (MC1–MC5), which bind with differing affinities to members of the MC hormone family [9]. These MC receptors share the structural features of the seven transmembrane, G-protein-coupled receptor (GPCR) superfamily. Each of the receptor subtypes has a characteristic tissue distribution. The MC1 receptor is located primarily in melanocytes, and is involved in melanocyte proliferation and melanogenesis [10]. The MC2 receptor, found in corticoadrenal tissue, binds ACTH and regulates both adrenal glucocorticoid and aldosterone production. The MC3 and MC4 receptors are both brain-specific, and whereas the function of MC3 receptor is unknown, the MC4 receptor has recently been shown to be involved in body-weight regulation [11]. The MC5 receptor is characterized by a broad distribution in tissues, including spleen, thymus, skin and bone marrow [12]. The physiological role of the MC5 receptor is unknown, although from its distribution, it has been hypothesized to be involved in the broad effects of α-MSH on peripheral tissues [12].

Members of the Janus kinase (JAK) family of intracellular tyrosine kinases have been shown to be involved in signal transduction from the cytokine receptor superfamily [13]. JAKs are known to associate with the membrane-proximal region of the ligand-bound cytokine receptor, leading to their phosphorylation on tyrosine, and subsequently, activation of kinase activity. Activated JAKs then phosphorylate the cytokine receptors, as well as other cytoplasmic proteins, known as the signal transducers and activators of transcription (STAT) proteins. This forms a unique signalling pathway shared by all members of the cytokine receptor family [13,14]. Recently, a report by Marrero et al. [15] provided evidence that the JAK/STAT pathway can also be stimulated by angiotensin II via its specific GPCR (AT_1). This work was the first to demonstrate that GPCRs are able to transduce signals via the intracellular phosphorylation pathways used by cytokine receptors.

This paper presents evidence that MC5 receptors are found on the surface of mouse pro-B-lymphocyte (Ba/F3) cells, and that α-MSH binding to Ba/F3 cells, human cultured lymphocyte (IM-9) cells or mouse L-cells expressing the cloned receptor induces the tyrosine phosphorylation and activation of the JAK2 signalling pathway.

MATERIALS AND METHODS

Cells, culture conditions and growth measurements

Ba/F3 mouse pro-B-lymphocyte cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) fetal bovine

Abbreviations used: ACTH, adrenocorticotropic hormone; FBS, fetal bovine serum; GPCR, G-protein-coupled receptor; IL, interleukin; JAK, Janus kinase; MC, melanocortin; MSH, melanocyte-stimulating hormone; [Nle^4, D-Phe^7]α-MSH, a potent MSH analogue; POMC, pro-opiomelanocortin; RT-PCR, reverse transcriptase-PCR; STAT, signal transducers and activators of transcription.

serum (FBS), 10 mM Hepes (pH 7.3), 10 μg/ml streptomycin sulphate, 10 units/ml penicillin and 5.0 % (v/v) WEHI cell condition medium (as a source of IL-3) in T75 tissue culture flasks at 37 °C and 5.0 % (v/v) CO_2. Cell growth was measured using the Alamar Blue assay system. Briefly, Ba/F3 cells were starved for 4 h by removal of WEHI. Cells were then incubated with the Alamar Blue redox indicator overnight in a 96-well plate in the presence of various concentrations of α-MSH, IL-3 or ACTH (4–10) (Sigma). $A_{570}-A_{600}$ was plotted as a function of MSH concentration, as an indication of cellular metabolic rate. IM-9 lymphocytes were cultured in RPMI 1640 medium/10 % v/v FBS/10 mM Hepes, pH 7.3, without antibiotics, under the same conditions as Ba/F3. No contamination of IM-9 cells by mycoplasma was detected. Mouse L-cells expressing the cloned MC-5 receptor were purchased from Ira Gantz (University of Michigan, MC, U.S.A.) [18] and were grown in Dulbecco's modified Eagle's medium (Gibco)/10 units/ml penicillin/ 10 μg/ml streptomycin sulphate/10 % (v/v) FBS.

MSH-binding analysis

Binding assays were performed in triplicate. 1×10^7 cells were incubated in RPMI 1640 medium/1.0 % (w/v) BSA (Sigma)/ 1 mg/ml bacitracin (Aldrich), with 40000 c.p.m. of ^{125}I-labelled [Nle^4, D-Phe^7]α-MSH (Peninsula), and various concentrations of unlabelled ligand in a 0.5 ml vol. for 2 h at room temperature. Non-specific binding was determined as total c.p.m. bound to cells in the presence of 1 μM [Nle^4, D-Phe^7]α-MSH. Following attainment of binding equilibrium, unbound ligand was separated from bound by centrifugation through a 20 % (w/v) sucrose cushion, and cell pellets were washed with PBS and counted in a γ-counter (Wallac).

mRNA preparation and transcript quantification

Poly(A)$^+$ mRNA was purified from Ba/F3 cells using the Straight A's mRNA Isolation System (Novagen). Reverse transcriptase-PCR (RT-PCR) analysis was performed using a reaction mixture containing 10 pg of template mRNA/20 mM Tris/HCl/100 mM KCl/0.1 mM EDTA/0.5 % Tween 20/50 % (v/v) glycerol/ 5 mM dithiothreitol/1.5 mM $MgCl_2$/10 mM each dNTP/20 μM upstream and downstream primers/5 units avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim)/0.5 units *Taq* DNA polymerase (Boehringer Mannheim). The reaction was first incubated in a Perkin–Elmer 9600 thermocycler at 50 °C for 30 min, followed by 25 cycles of 94 °C for 45 sec, 50 °C for 45 sec and 68 °C for 45 sec. Products were electrophoresed on a 1 % (w/v) agarose gel, and stained with ethidium bromide. PCR reactions using plasmids encoding each of the five MC receptors as template (purchased from Ira Gantz, University of Michigan, MC, U.S.A.), and each specific primer, were performed under identical cycling conditions in a reaction containing 50 mM KCl/10 mM Tris/HCl (pH 8.3)/2 mM $MgCl_2$/10 mM each dNTP/50 ng of plasmid template/20 μM upstream and downstream primers/2.5 units *Taq* DNA polymerase. Alternate upstream primers specific for each MCR are as follows (5′ → 3′): MC1, TGCTGCTGGAGGTGGGCATCCTGGTGGCCAGAGTG; MC2, TGTTCAGAAACATGGGTTATCTTAAGCCTCGTGG; MC3, CCGTGATCAACAGCGACTCCCTGACCTTGGAGGAC; MC4, CACCCTATTAAACAGTACAGATACGGATGCACAGAGTTTCACAGTGAA; MC5, ACTTGCTAAATAATAAACACCTGGTGATAGCCG. The downstream degenerate primer is CTCTTGGCTGCAGANNGCNTAGATGAGNGGNTC, where N represents any nucleotide.

Immunoprecipitation, nuclear extraction and Western blotting

Following induction with α-MSH, approx. 2×10^7 cells (Ba/F3 or IM-9) were harvested, washed with ice-cold PBS containing 1 mM Na_3VO_4, and lysed by gentle agitation in lysis buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/1.0 % (w/v) Nonidet P40/0.5 % (w/v) sodium deoxycholate/1 mM Na_3VO_4/0.2 mM PMSF/1 μM pepstatin/1 μg/ml aprotinin/0.5 mM EDTA]. In the case of L-cells, cells were grown to confluence in a T75 flask, deprived of serum overnight, washed once in PBS, and induced with 10 nM α-MSH in Dulbecco's modified Eagle's medium for the indicated times; cells were then washed once in ice-cold PBS containing 1 mM Na_3VO_4, then scraped in 1 ml of lysis buffer. Following centrifugation at 14000 ***g*** for 10 min at 4 °C, all extracts were precleared for 3 h by incubation with protein A agarose (Boehringer Mannheim), then incubated with 5 μl of either anti-(JAK2 antiserum) (Upstate Biotechnology, Inc.), anti-(phosphotyrosine monoclonal antiserum) (Gibco) or anti-(STAT1–5 antiserum) (Upstate Biotechnology, Inc.) at 4 °C overnight. The immune complex was bound to protein A for 3 h at 4 °C, collected by centrifugation, and washed four times in lysis buffer. Samples were heated to 100 °C for 3 min in SDS sample buffer. Proteins were separated by SDS/PAGE on a 4–12 % acrylamide gradient and transferred to a PVDF membrane (Boehringer Mannheim). Blots were probed with either anti-phosphotyrosine conjugated to horseradish peroxidase (Boehringer Mannheim) and developed with enhanced chemiluminescence, or probed with anti-JAK2 followed by a goat anti-(rabbit IgG) conjugated to alkaline phosphatase (Sigma) and developed with an alkaline phosphatase detection system (Boehringer Mannheim). Following hybridization, some blots were stripped before re-hybridization with a second antibody by incubation in Tris-buffered saline containing 100 mM 2-mercaptoethanol and 2 % (w/v) SDS for 30 min at 50 °C, followed by two 15 min washes in Tris-buffered saline.

Nuclear extracts were prepared by centrifuging cell extracts in lysis buffer for 10 min at 4 °C at 1000 ***g***; pellets were then resuspended in nuclear extraction buffer [20 mM Hepes/0.4 mM NaCl/1 mM EDTA/25 % (v/v) glycerol/1 mM dithiothreitol/ 1 mM Na_3VO_4/0.2 mM PMSF/1 μM pepstatin/1 μg/ml aprotinin] and incubated on ice for 30 min followed by a 10 min spin at 10000 ***g*** at 4 °C. The nuclear protein-containing supernatant was quantified using the spectrophotometric ESL protein assay (Boehringer Mannheim).

RESULTS AND DISCUSSION

Ba/F3 cells express MC receptors

In order to determine whether the mouse pro-B-lymphocyte cell line Ba/F3 expresses specific MC-binding activity, the affinity for α-MSH was determined using the iodinated α-MSH analogue ^{125}I-[Nle^4, D-Phe^7]α-MSH as a radioligand tracer in a whole-cell equilibrium-binding assay. [Nle^4, D-Phe^7]α-MSH is a potent α-MSH analogue with a substitution of norleucine at position 4 and D-phenylalanine at position 7 of α-MSH, resulting in a molecule with prolonged biological activity and resistance to degradation by serum enzymes, used routinely in studies of α-MSH binding [19]. In the competition curve shown in Figure 1, bound ^{125}I-[Nle^4, D-Phe^7]α-MSH is specifically competed away by increasing concentrations of either [Nle^4, D-Phe^7]α-MSH or α-MSH, with apparent IC_{50} values of $2.0 +/- 1.0$ nmol/l and $7.2 +/- 1.0$ nmol/l respectively, which is consistent with known affinities of the MC receptors. Data are consistent with a single-site model of whole-cell ligand binding [16].

In order to determine which, if any, of the previously charac-

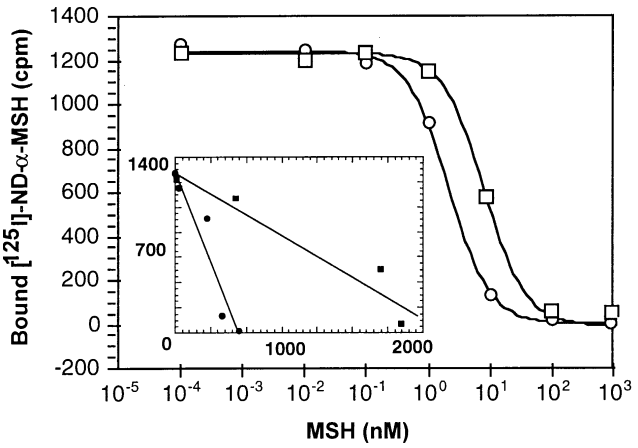

Figure 1 Competition curve of α-MSH binding to Ba/F3 cells

^{125}I-[Nle^4, D-Phe^7]α-MSH, bound to whole Ba/F3 cells, is competed away by increasing concentrations of unlabelled [Nle^4, D-Phe^7]α-MSH (open circles) or α-MSH (open squares). Each point is the average of triplicate measurements. Inset: bound versus bound × inhibition plot of the same data, in which bound ^{125}I-[Nle^4, D-Phe^7]α-MSH (c.p.m.) is plotted against the concentration of inhibitor (nM) multiplied by bound ^{125}I-[Nle^4, D-Phe^7]α-MSH (c.p.m.), such that the slope is the negative reciprocal of the IC_{50}. IC_{50} values for [Nle^4, D-Phe^7]α-MSH and α-MSH are 2.0 ± 1.0 nmol/l and 7.2 ± 1.0 nmol/l respectively.

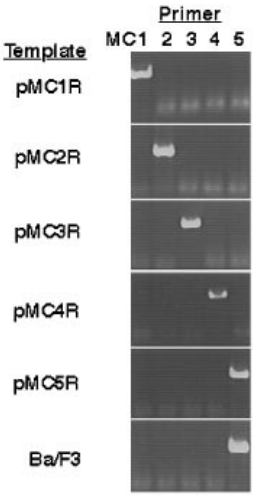

Figure 2 Ba/F3 lymphocytes express MC5 receptor subtype

Shown are the results of RT-PCR analysis using Ba/F3 mRNA as template (bottom panel) and upstream PCR primers specific for each receptor subtype. Only the MC5-specific primer amplifies a product of predicted size (band shown is 630 bp). The five upper panels are PCR reaction products from reactions using plasmids encoding each of the five known subtypes of MC receptors (pMC1–5R) as template, the selective MC receptor specific primers (MC 1–5), and a common downstream primer. Smaller fuzzy bands represent primer dimers.

terized MC receptors are expressed in these cells, transcript levels for the five known MC receptors were determined by RT-PCR using oligonucleotides specific for each of the five known MC receptors. mRNA was isolated from Ba/F3 cells, and used as a template with alternate upstream primers specific for each MC receptor, and a single downstream primer identical to a conserved region within the MC receptor gene family. The predicted size of the RT-PCR-amplified MC receptor is 630 bp. As shown in Figure 2, only the MC5-receptor-specific primer is able to amplify

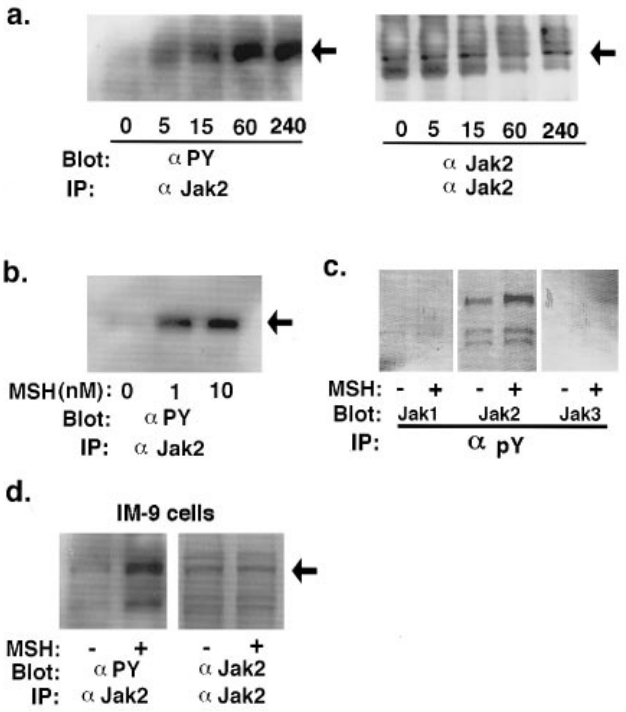

Figure 3 Stimulation of JAK2/STAT1 by α-MSH in lymphocytes

Cells were treated with or without α-MSH, immunoprecipitated with either anti-JAK2 or anti-phosphotyrosine, and Western-blotted with either anti-JAK2 or anti-phosphotyrosine. (**a**) Ba/F3 cells were treated for 0, 5, 15, 60 or 240 min with 10 nM α-MSH, immunoprecipitated with anti-JAK2, and blotted with either anti-phosphotyrosine or anti-JAK2, as indicated. In all panels, the arrow indicates the 130 kDa JAK2 band. Multiple bands found in the JAK2-immunoprecipitated material were consistently observed. (**b**) Ba/F3 cells were treated with 0.1 or 10 nM α-MSH for 15 min, immunoprecipitated with anti-JAK2, and blotted with anti-phosphotyrosine. (**c**) Ba/F3 cells were treated with or without 10 nM α-MSH, immunoprecipitated with anti-phosphotyrosine, and blotted with either of anti-JAK1 or anti-JAK2, or anti-JAK3. (**d**) Human cultured IM-9 lymphocytes were treated with or without 10 nM α-MSH for 60 min, immunoprecipitated with anti-JAK2, and blotted with either anti-phosphotyrosine or anti-JAK2 antisera.

a 630 bp band using Ba/F3 mRNA as template, demonstrating that these cells express only the MC5 receptor subtype. As specificity controls, each oligonucleotide (MC 1–5) was tested in PCR reactions under similar conditions using plasmid templates encoding each MC receptor (pMC1–5R). Each primer will only amplify a product in reactions using a template for which it is specific. Together, these results demonstrate that Ba/F3 cells express the MC5 receptor.

Effect of α-MSH on JAK2 signal transduction

To investigate whether α-MSH stimulates JAK2 phosphorylation, Ba/F3 cells were exposed to α-MSH, and JAK2 phosphorylation was measured by immunoprecipitating cell lysates with anti-JAK2, followed by Western blotting with anti-phosphotyrosine or anti-JAK2 antisera. One observes a time- (Figure 3a) and concentration- (Figure 3b) dependent increase in JAK2 tyrosine phosphorylation. Activation of JAK2 by α-MSH in Ba/F3 lymphocytes is both rapid and sustained, with visible phosphotyrosine accumulation present after only 5 min, with continued accumulation up to 240 min. Overall, JAK2 protein levels are unaffected by α-MSH incubation (Figure 3a).

JAK2 activation was also demonstrated by immunoprecipitating Ba/F3 cell lysates with anti-phosphotyrosine, and probing

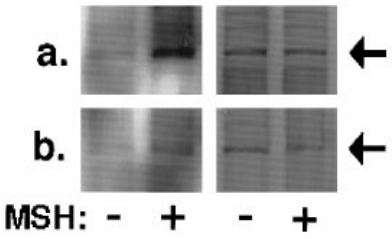

Figure 4 JAK2 activation by cloned MC receptors

Mouse L-cells stably expressing the MC5 receptor were stimulated with 10 nM α-MSH for 15 min. (**a**) Western blot of JAK2-immunoprecipitated material from MC5-expressing cells probed with anti-phosphotyrosine (left panel) or anti-JAK2 (right panel). (**b**) Same as (**a**), but from MC1-receptor-expressing L-cells. Arrow indicates the migration of the 130 kDa band found in the JAK2 immunoprecipitate. This blot is representative of four independent experiments.

three blots in parallel with either anti-JAK1, anti-JAK2 or anti-JAK3 antisera (Figure 3c). Whereas incubation with α-MSH induced JAK2 tyrosine phosphorylation over basal levels, no induction of either JAK1 or JAK3 phosphate levels was observed, and neither could basal levels of these kinases be detected in this assay. A low level of basal JAK2 phosphorylation is usually observed in non-stimulated cells (see Figure 3c and 3d); this may be the result of residual stimulation by other growth factors carried over from the serum-containing growth medium, or a reaction to serum starvation during the procedure.

In order to determine whether JAK2 phosphorylation by α-MSH is a general phenomenon of lymphocyte cells, human IM-9 lymphocytes were likewise induced with α-MSH, immunoprecipitated with anti-JAK2, and blotted with anti-phosphotyrosine or anti-JAK2. One also observes an α-MSH-mediated activation of JAK2 phosphorylation in these cells (Figure 3d), indicating that IM-9 cells also respond to α-MSH via JAK2 phosphorylation, and indeed, this may be a phenomenon common to all lymphocytes. Identical results were obtained when IM-9s were induced with [Nle^4, D-Phe^7]α-MSH (results not shown).

Activation of JAK2 by cloned MC receptors

In order to determine whether α-MSH stimulation of the JAK pathway is receptor-mediated, and whether this process does not involve lymphocyte-specific processes, JAK2 activation was assayed in mouse L-cells (a fibroblast-like cell line) stably expressing either the MC5 or MC1 receptor. Phosphorylated JAK2 was observed in mouse L-cells stably expressing the cloned MC5 receptor following a 15 min incubation with 10 nM α-MSH (Figure 4). JAK2 levels in MC1-receptor-expressing L-cells were also stimulated to slightly above-basal levels by α-MSH. No tyrosine phosphorylation of JAK2 is observed upon stimulation of parental L-cells lacking the receptor (results not shown). Thus the cloned MC receptor is able to stimulate JAK2 phosphorylation in a heterologous system, suggesting that this interaction is a direct receptor-mediated event, and not mediated by lymphocyte-specific secondary signals.

Effect of α-MSH on the STAT pathway

The paradigm of cytokine signalling is that tyrosine phosphorylation activates members of the JAK family, which then phosphorylate the membrane-bound receptors, and also members of the family of latent cytoplasmic transcription factors known as STATs. In order to confirm that α-MSH stimulation activates

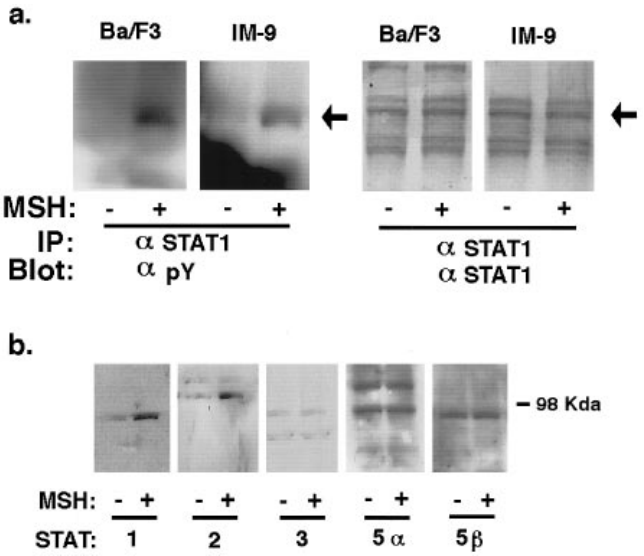

Figure 5 Activation of STAT proteins by α-MSH

(**a**) Cells were stimulated with 10 nM α-MSH for 60 min; cell extracts were immunoprecipitated with anti-STAT1 and blotted with anti-phosphotyrosine (left panel) or anti-STAT1 (right panel). The migration of the 91 kDa STAT1 product is indicated by an arrow. (**b**) Nuclear translocation of STATs. Cells were lysed as for (**a**), nuclear extracts were prepared and 20 μg of protein per lane was separated by SDS/PAGE and Western-blotted with specific antisera corresponding to a variety of STAT proteins.

the well-established JAK/STAT pathway, Ba/F3 and IM-9 lymphocytes were assayed for the activation of p91-STAT1 tyrosine phosphorylation (Figure 5a). Treatment with 10 nM α-MSH induced STAT 1 phosphorylation in both cell types (Figure 5a). This result suggests that α-MSH signals through STAT1, probably as a result of JAK2 activation.

STAT protein phosphorylation by growth factors and cytokines results in the translocation of the phosphorylated STATs to the nucleus. Thus an alternative way to assay for the activation of STAT1 by α-MSH is to examine relative STAT1 levels present in the nucleus before and after stimulation by α-MSH. To this end, Ba/F3 cells were stimulated for 60 min by 10 nM α-MSH, and nuclear extracts were isolated. Nuclear extracts were quantified, and equivalent amounts were run on a gel and blotted with anti-STAT1 antiserum. Figure 5(b) shows the accumulation of STAT1 in nuclear extracts in response to α-MSH stimulation. This assay was employed to examine the activation of other STAT proteins. This assay demonstrates a clear nuclear accumulation of STAT2 in response to α-MSH. STATs 3, 5α and 5β were present in the nucleus, but in equal amounts in both stimulated and unstimulated lanes. No STAT4 protein could be detected in either lane (results not shown).

Effect of α-MSH on B-cell proliferation

To examine the potential role of α-MSH in B-lymphocyte function, Ba/F3 cell proliferation was measured as a function of α-MSH concentration. The growth curves of Figure 6(A) demonstrate that α-MSH stimulates the growth of Ba/F3 lymphocytes, which demonstrates a specific cellular effect of α-MSH on these cultured cells. [Nle^4, D-Phe^7]α-MSH is more potent at stimulating cell growth (Figure 6A), which is consistent with its higher binding affinity. Growth induction by the MC peptide fragment ACTH (4–10) could not be measured in this assay,

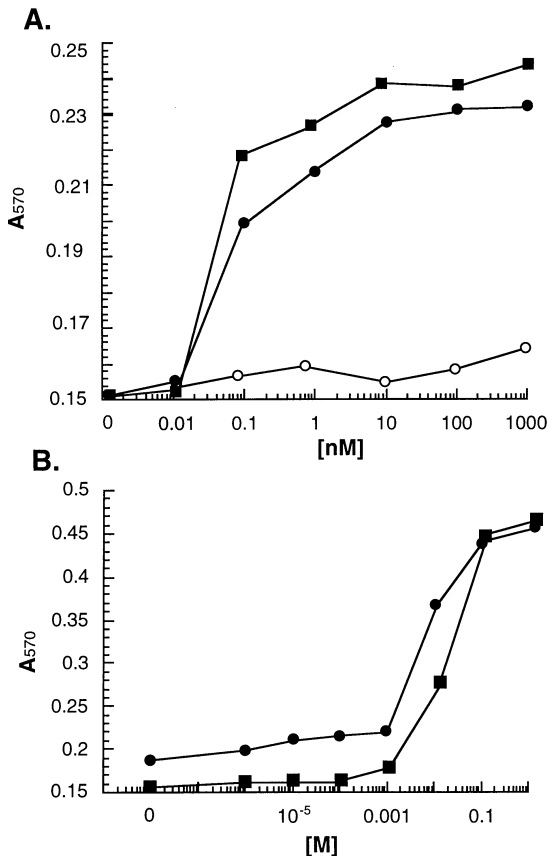

Figure 6 α-MSH induces B-cell proliferation

(**A**) Ba/F3 cells were deprived of IL-3 for 4 h, then assayed for growth in the presence of increasing concentrations of α-MSH (closed circles), [Nle^4, D-Phe^7]α-MSH (squares), or ACTH (4–10) (open circles). (**B**) Starved cells were grown in the presence of increasing concentrations of IL-3 in the presence (circles) and absence (squares) of 10 nM α-MSH. Points are averages of duplicate measurements.

consistent with earlier studies demonstrating that ACTH (4–10) has no effect on MC5 receptor signal transduction [12,18]. A larger effect on cell proliferation can be demonstrated by titrating the concentration of IL-3, a growth factor necessary for Ba/F3 cells, in the presence and absence of 10 nM α-MSH (Figure 5B). Under these conditions, one observes a left-shift in the EC_{50} value of approximately four-fold (from 20 nM to 5 nM). This result demonstrates that α-MSH is able to promote growth of Ba/F3 cells on its own, and also enhances cell proliferation *in vitro* in the presence of IL-3, suggesting that the growth *in vivo* of B-cells may be positively effected by α-MSH under physiological conditions.

These data are in agreement with results of other workers, namely Alvarez-mon, Kehrl and Fauci [8], who reported that an MC (ACTH) induces a clear synergistic effect with IL-2 in the blastogenesis of B-cells of about 2.5–3-fold, although in their study, ACTH did not activate B-cell proliferation by itself. Activation of proliferation in B-cells by MCs is consistent with the observed stimulation of JAK2, and suggests that activation of JAK2 and STAT proteins results directly in the induction of mitogenesis [17]; however, the possibility that the observed proliferative response is epiphenomenal to JAK2 activation cannot be ruled out.

The present work provides evidence that α-MSH signals through MC5 receptors on the surface of B-lymphocytes, which leads to the tyrosine phosphorylation and activation of the JAK2 cytoplasmic kinase. This effect is seen in mouse pro-B-lymphocyte (Ba/F3) cells, in human lymphocyte (IM-9) cells, as well as in mouse L-cells stably expressing the MC5 receptor. Activation of JAK2 by α-MSH in Ba/F3 lymphocytes occurs rapidly and can be observed after only 5 min (Figure 4), with activation sustained even after 4 h.

One can envision several mechanisms whereby α-MSH binding to MC5 receptor would lead to JAK2 activation. JAK2 tyrosine phosphorylation in response to α-MSH-receptor ligand occupancy may be the result of a direct physical interaction between the receptor and JAK2, analogous with cytokine signalling. Activation of the MC5 receptor may also lead to the dimerization and activation of an endogenous cytokine receptor present on the Ba/F3 cell membrane. Alternatively, occupation of MC5 receptors may trigger, as yet, unidentified downstream events which result, ultimately, in JAK2 activation. For example, the G-protein-mediated activation of protein kinase A (PKA) is known to affect signalling from receptor tyrosine kinases at the level of mitogen-activated protein kinase activation. Perhaps PKA similarly phosphorylates JAK2. Finally, α-MSH may lead to the activation or secretion of other factors, which in turn activate JAK2; in T-cells, for instance, α-MSH stimulation is known to result in secretion of IL-10, a cytokine known to signal through JAK and STAT. However, JAK2 activation is also observed in mouse L-cells stably transfected with the MC5 receptor. The fact that the MC5 receptor signals through JAK2 in a heterologous cell system suggests that activation of JAK2 is direct, or at least is not mediated by lymphocyte-specific processes, e.g. IL secretion, etc. Further studies on the interaction of MC5 receptor with JAK2 await the development of an MC5-receptor-specific antibody.

Activation of the JAK kinase family is generally considered to be a pathway involved in cytokine and interferon-mediated signalling, although the activation of JAK2 by a GPCR is not without precedence: it has been shown that the angiotensin II AT_1 receptor signals through JAK2 and STAT1 [15]. The mechanism of this activation is thought to be analogous with that of cytokine signalling, and may be involved in growth responses in vascular smooth-muscle cells by angiotensin. Perhaps, as more GPCRs are investigated for JAK2 phosphorylating activity, it will turn out to be a more significant means of GPCR signalling than was once thought.

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