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Progesterone Increases csk Homologous Kinase in HMC-1^{560} Human Mast Cells and Reduces Cell Proliferation

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Abstract Mast cells proliferate in vivo in areas of active fibrosis, during parasite infestations, in response to repeated immediate hypersensitivity reactions and in patients with mastocytosis. We investigated how progesterone reduces the proliferation of HMC-1^{560} mast cells that proliferate spontaneously in culture. Cells were incubated with 1 μM to 1 nM progesterone for 24–48 h. Progesterone (1 μM) reduced the spontaneous proliferation of HMC-1^{560} mast cells to half that of cells cultured without hormone. [^{3}H] thymidine incorporation was only 50% of control; there were fewer cells in G2/M and more cells in G0/G1. The amounts of phospho-Raf-1 (Tyr 340–341) and phospho-p42/p44 MAPK proteins were also reduced. In contrast progesterone had no effect on MAP kinase-phosphatase-1. The Raf/MAPK pathway, which depends on Src kinase activity, is implicated in the control of cell proliferation. HMC-1^{560} cells incubated with the tyrosine kinase inhibitor PP1 proliferated more slowly than controls and had less phospho-Raf-1 (Tyr 340–341) and phospho-p42/p44 MAPK. The Csk homologous kinase (CHK), an endogenous inhibitor of Src protein tyrosine kinases, was also enhanced in progesterone-treated cells. In contrast, progesterone had no effect on the growth of cells transfected with siRNA CHK. We conclude that progesterone increases the amount of csk homologous kinase, which in turn reduces HMC-1^{560} mast cell proliferation. This effect parallels decreases in the phosphorylated forms of Raf-1 and p42/44 MAPK, as their production depends on Src kinase activity. J. Cell. Biochem. 102: 1271–1280, 2007.

Key words: proliferation; progesterone; mast cells; Src/MAPK; csk homologous kinase

Mast cells are key components of innate immunity. They are widely distributed throughout vascularized tissues, certain epithelia and various mucosal sites, and their accumulation in tissues is associated with many physiological functions. They do not usually proliferate in humans, except in areas of active fibrosis, during parasite infestations, and in response to repeated immediate hypersensitivity reactions, e.g., in allergic rhinitis and asthma. Lastly, mastocytosis is a heterogeneous group of disorders characterized by the accumulation of mast cells in organs, due in part to the abnormal proliferation of mast cells that is often associated with c-kit receptor dysfunction [Lennartsson et al., 2005]. Patients with systemic mastocytosis suffer from flushes, itching and osteoporosis due to the abnormal proliferation of mast cells in the skin and bone marrow. Thus there are more mast cells in the dermis of patients with systemic mastocytosis than in the dermis of patients with atopic dermatitis [Brockow et al., 2002]. Some pediatric cutaneous forms of mastocytosis regress at puberty, suggesting that sex hormones suppress mast cell functions [Marone et al., 2001; Castells, 2004]. A reciprocal relationship between sex steroids and the immune system has been evident for several years, and there is now evidence indicating that ovarian hormones influence the distribution and function of mast cells [Grossman, 1984; Beagley and Gockel, 2003]. Progesterone is produced by both the ovary and the placenta. Its synthetic forms,

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progestins, are used in contraception and to treat dysfunctional uterine bleeding. They are also used to minimize the risk of uterine cancer associated with estrogen replacement therapy and are widely employed to treat metastatic endometrial and breast cancers. Progesterone is not only important for reproduction and pregnancy. It also controls immune functions in women, and progesterone receptors have recently been identified in mast cells in the upper airways and bladder [Letourneau et al., 1996; Zhao et al., 2001]. Progesterone can either stimulate or inhibit cell proliferation, depending on the cell type, duration of the response, and interaction with other hormones or growth factors. Progesterone decreases estrogen-induced cell proliferation in the uterus and inhibits the growth of the human endometrium [Graham and Clarke, 1997], breast cancer cells [Dai et al., 2002; Gizard et al., 2005] and activated T lymphocytes [Vassiliadou et al., 1999].

We have used a variant subline of the HMC-1 cell line, HMC-1^{560} [Butterfield et al., 1988; Sundstrom et al., 2003] that has a point mutation in the juxtamembrane domain of the *c-kit* proto-oncogene. The c-kit receptor is a member of the receptor tyrosine kinase (RTK) subclass III family. It activates the Raf-MEK (MAPK/ERKkinase)-ERK MAPK cascade [Marshall, 1995; Seger et al., 1995]. This well-described pathway is implicated in the control of mast cell proliferation. It depends greatly on tyrosine phosphorylation, starting with RTK transphosphorylation, recruitment of the Src homology 2 domain containing adaptor proteins Shc and Grb2, and the activation of Ras and Raf. These in turn activate MEK, a dual-specificity kinase that is specific for ERK. Phosphorylated ERK moves to the nucleus where it activates the cell cycle machinery [Kerkhoff and Rapp, 1998]. This report describes the action of progesterone on the spontaneous proliferation of HMC-1^{560} mast cells. Cells grown in medium containing progesterone had subnormal amounts of active phosphorylated Raf-1 and p42/44 MAP kinases and a parallel increase in the amount of csk homologous kinase (CHK), an endogenous inhibitor of the Src-family protein tyrosine kinases.

MATERIALS AND METHODS

Cell Line

All studies were done with the variant of the HMC-1 cell line, subline HMC-1^{560}, that was originally obtained from a patient with mast cell leukemia [Butterfield et al., 1988; Sundstrom et al., 2003]. HMC-1^{560} cells have a point mutation in the juxtamembrane domain causing Val-560 to be replaced by Gly. Substitution of glycine for valine 560 in human c-Kit results in constitutive activation of c-Kit, which is the major cause of mast cell spontaneous proliferation [Kitamura et al., 1995]. Cells were grown at 37°C with 5% CO_2 in MEM without phenol red (Sigma–Aldrich, St-Quentin Fallavier, France) supplemented with 10% dialysed fetal calf serum (FCS) from Gibco BRL (Invitrogen, Cergy Pontoise, France). HMC-1^{560} cells proliferated spontaneously under these experimental conditions.

Reagents

Progesterone and the MEK inhibitor PD98059 were purchased from Sigma–Aldrich France (St-Quentin Fallavier, France). PP1, a specific inhibitor of Src kinases, was purchased from Biomol (Tebu, Le Perray en Yveline, France).

Proliferation Assay

Cells (1×10^4 cells/well in 200 μl medium) were seeded on 96-well plates in triplicate. [^{3}H] thymidine (0.5 μCi/well) (Perkin-Elmer® Life and Analytical Sciences, Boston, USA) was added to each well overnight. Cells were immobilized on glass fibre filters (Wallac, Turku, Finland) using a Harvester 96 (Tomtec Inc. Hamden, USA). The incorporated radioactivity was determined by scintillation counting (1205 Betaplate, LKB Wallac).

Annexin V Staining

HMC-1^{560} cells were stained with annexin V using the TACS Annexin V-FITC apoptosis detection kit (R&D Systems Europe, Lille, France) according to the manufacturer's instructions. Briefly, cells were washed in phosphate-buffered-saline (PBS) and incubated in the dark for 15 min at room temperature in 100 μl binding buffer (100 mM HEPES pH 7.4, 1.5 M NaCl, 50 mM KCl, 10 mM $MgCl_2$, 18 mM $CaCl_2$) containing 10 μl propidium iodide and 1 μl annexin V conjugated to fluorescein isothiocyanate (FITC), then analyzed on a flow cytometer FACScan (Becton Dickinson). Cells treated with 100 nM dexamethasone were used as a positive control according to the manufacturer's instructions.

Cell Cycle Analysis

Cell cycle analyses were performed using propidium iodide (PI) staining followed by FACS analysis. Briefly, suspensions of 1×10^5 cells were centrifuged at 400 g for 10 min. The resulting pellets were washed in ice-cold PBS and fixed in 1 ml ice-cold 70% ethanol for 20 min on ice. The cells were washed in PBS, treated with 40 μg/ml RNase at 37°C for 30 min, and then washed in PBS. The cells were stained with 20 μg propidium iodide in 1 ml PBS overnight at 4°C. Fluorescence was measured with a FACS-Caliber flow cytometer (Becton Dickinson, Le Pont de Claix, France), and analyzed using Cellquest software for the distribution of cells in the various phases of the cell cycle.

RT-PCR Procedures

Total RNA was extracted from cultured cells using the SV total RNA isolation system (Promega France, Cergy-Pontoise). The mRNA was transcribed into cDNA by RT with random hexamers as primers and Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, 69260 Charbonnières-les-bains, France). PCR products were quantified and validated as previously described [Zou et al., 1995; Taupeau et al., 2003]. Samples of cDNA were standardized by measuring β-actin cDNA by competitive PCR. CHK cDNA was assayed using an appropriate PCR protocol on preparations containing the same amount of β-actin cDNA. The number of cycles was determined during the amplification phase of DNA. The oligonucleotides used for PCR amplification were: CHK-F 5′-ttgctcagtgtgcctctcac-3′ and CHK-R 5′-cgcatttggtgatacactgg-3′ (355–571). The hybridization temperature was 62°C. PCR products (216 bp) were visualized on 2% agarose gel electrophoresis under UV. The intensity of the bands was measured with FotoLook SA 2.0 software (AGFA-Gevaert, Munich, Germany).

Western Blot Analysis

Cells (1×10^6) were sonicated on ice (4×5 s) at 40 kHz and stored in 1× Laemmli buffer (Bio-Rad, Ivry, France). Cell lysates (60 μg protein) were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 10% gels) and transferred to nitrocellulose membranes (Hybond-ECL, Amersham Pharmacia Biotech Europe). The proteins were probed with anti-beta-actin, anti-MAPK phosphatase-1 (MKP-1), anti-Lsk (CHK/MATK), anti-phospho-Raf-1 ($Tyr^{340/341}$) and anti-PR antibodies (Santa Cruz Biotechnology, Inc., Tebu, Le Perray-en-Yvelines, France), anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}), anti-p44/42 MAPK (Cell Signaling Technology™, Ozyme, Saint Quentin-en-Yvelines, France), anti-c-Raf-1 (BD Biosciences, Le Pont-de-Claix, France). Primary antibodies were visualized with peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat IgG (Santa Cruz, Tebu, Le Perray-en-Yveline, France) followed by chemiluminescence using the ECL™ kit (Amersham, Saclay, France). Exposed films were scanned on an Imstar densitometer and the intensity of the signals was measured using FotoLook SA 2.0 software.

RNA Interference Experiments

Lsk siRNA (h) was provided by Santa Cruz Biotechnology, Inc., Tebu, Le Perray-en-Yvelines, France. It is a specific 20–25 nt siRNA designed to knock out CHK gene expression. Non-targeting 20–25 nt siRNA was used as a negative control (SiRNA-A) (Santa Cruz Biotechnology INC). $HMC\text{-}1^{560}$ cells were cultured for 24 h and then transfected with siRNAs using the nucleofection Amaxa system (Amaxa, Cologne, Germany). Cells (2×10^6) were suspended in 100 μl nucleofector kit V solution and the suspension mixed with 0.6 μg of control siRNA-A or Lsk (CHK) siRNA (h). This mixture was placed in the Amaxa Nucleofector apparatus (Amaxa). Cells were transfected using the T-20 pulsing parameter and then immediately transferred to wells containing 1ml of warm (37°C) culture medium in 12-well plates. The efficiency of transfection was assessed using the green fluorescent protein (GFP)-reporter vector in the Amaxa kit. The production of GFP by $HMC\text{-}1^{560}$ cells was determined at 4 h and 16 h after transfection by FACS analysis using FACSCan (BD Biosciences). Twenty-four hours later, the cells were washed, disrupted and the proteins extracted.

Statistical Analysis

Arithmetic means, medians, standard deviations, standard errors, and statistical analyses were done using PRISM® software. Differences between groups were calculated using one-way analysis of variance (ANOVA) for repeated measures followed by Tukey's multiple comparison test. Differences between two paired

groups were calculated using paired t-test and comparison between two unpaired groups were calculated using unpaired t test at $P < 0.05$. Significance was defined as a two-tailed P value of <0.05* or <0.01**.

RESULTS

Progesterone and Cell Proliferation

Western blotting showed that HMC-1^{560} cells contained the A isoform (94 kDa) and B isoform (114 kDa) of the conventional progesterone receptor (PR) (Fig. 1A).

The effects of progesterone on the spontaneous proliferation of HMC-1^{560} cells were determined by measuring the incorporation of [^{3}H]-thymidine into cells cultured for 24 h and 48 h with zero, 1 nM, 10 nM, 100 nM, or 1 μM progesterone. The results of three independent experiments are expressed as a percentage of the control (Fig. 1B). Incubation with 1 μM progesterone for 24 h produced around 50% inhibition, which continued for 48 h. There was a significant difference between cells treated with 1 μM progesterone and controls ($P < 0.01$, n = 3). Progesterone vehicle (1% ethanol) had no effect.

We also examined the effect of 1 μM progesterone on cell viability using annexin V-propidium iodide double staining. Cells treated with 100 nM dexamethasone were used as a positive control. We detected no significant increase in apoptotic and dead cells after progesterone treatment while dexamethasone increased the number of apoptotic cells (Table I).

Progesterone and Cell Cycle Arrest in G0/G1

The DNA profiles of control cells and cells incubated with 1 μM progesterone for 24 h (Fig. 2) were compared by measuring the percentages of cells in G0/G1, S and G2/M

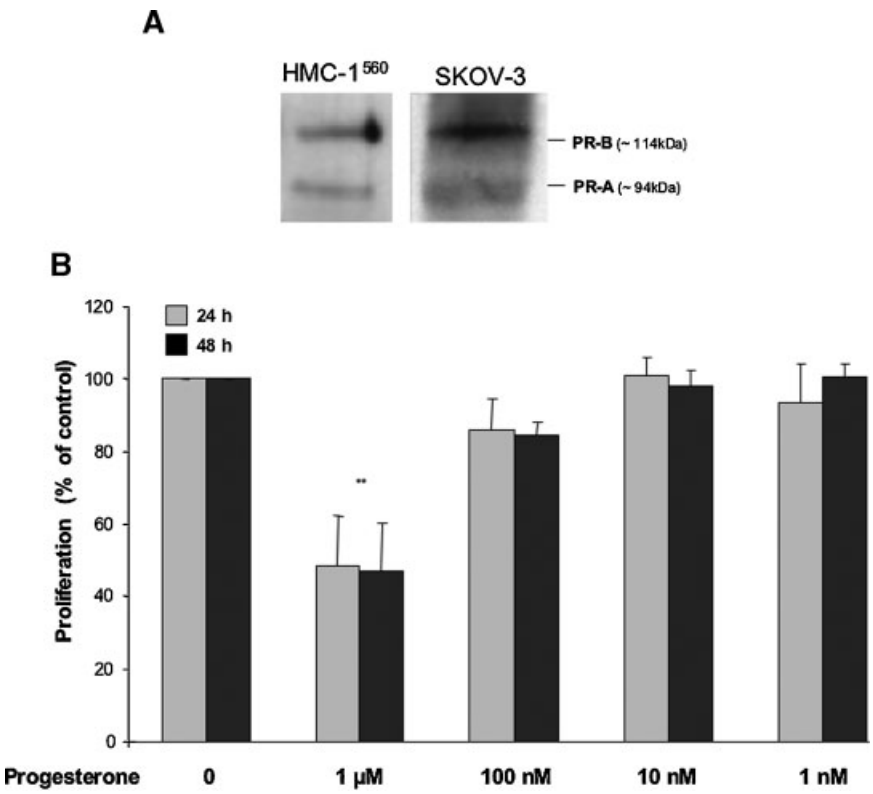

Fig. 1. Progesterone receptors and the effect of progesterone on the proliferation of HMC-1^{560} cells. **A**: Western blotting for progesterone receptors (PR) in HMC-1^{560} cells revealed the A isoform (PR-A) at 94 kDa and the B isoform (PR-B) at 114 kDa. A positive control was run with SKOV-3 ovarian cancer cells. **B**: cells were cultured with 1 μM, 100 nM, 10 nM or 1 nM progesterone for 24 h and 48 h. Controls was run without progesterone. Proliferation was measured by [^{3}H]-thymidine incorporation, and expressed as a percentage of control. Histograms show data from three independent experiments. Differences between treated and untreated cells were calculated using ANOVA for repeated measures followed by Tukey's multiple comparison test. Progesterone (1 μM) significantly inhibited the proliferation of HMC-1^{560} mast cells, $^{**}P < 0.01$, n = 3.

TABLE I. Apoptosis in Progesterone-Treated Cells

	Percentages of cells (mean ± S.E.M)		
	Viable (annexin V−/PI−)	Apoptotic cells (annexin V+/PI−)	Dead cells (annexin V+/PI+)
Control	63.7 ± 3	23.35 ± 8.35	12.95 ± 5.35
Progesterone (1 μM)	64 ± 1.1	23.85 ± 5.45	12.15 ± 4.35
Dexamethasone (100 nM)	38.5 ± 10.1	28.6 ± 5.2	32.95 ± 15.25

(Table II). Cells incubated with 1 μM progesterone for 24 h showed an increase in cells in G0/G1 (mean ± S.E.M = 55.4% ± 6.8 versus controls = 38% ± 4.4, n = 4). In parallel, the percentages of cells in G2/M were lower in progesterone-treated cells than in untreated cells.

Impact of the Src/Raf/MAPK Pathway on Spontaneous HMC-1^{560} Cell Proliferation

We investigated the impact of activating the Src/Raf/MAPK pathway on the spontaneous proliferation of HMC-1^{560} cells using PD98059 (20 μM), a MEK inhibitor, and PP1 (500 nM), a specific Src kinase inhibitor. Both inhibitors reduced the cell proliferation measured by [^{3}H]-thymidine incorporation (Fig. 3A), while inhibitor vehicle (1% DMSO) had no effect. Western blotting also revealed that PP1 decreased the amounts of the phosphorylated forms of Raf-1 and p42/44 MAPK, while PD98059 only decreased the amount of phosphorylated p42/44 MAPK (Fig. 3B).

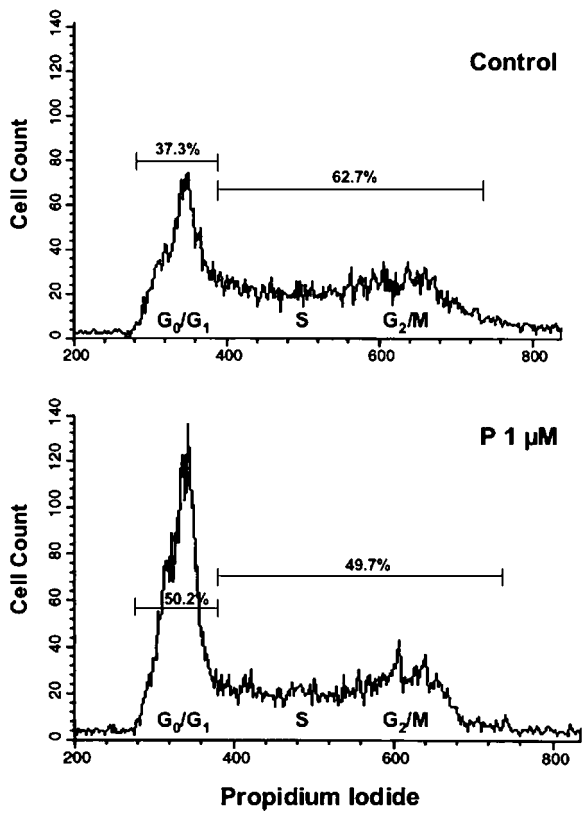

Fig. 2. Effect of progesterone on the cell cycle. Cell cycles were analyzed using propidium iodide (PI) staining and FACS analysis. DNA profiles were analyzed under basal conditions (control) and after incubation with 1 μM progesterone (P 1 μM) for 24 h. Diagrams are those of one representative experiment of 4. 62.7% untreated cells progressed in the cell cycle (S and G2/M) beyond G1 (37.3% in G1), while only 49.7% of cells incubated with P did so (50.2% in G1).

Progesterone and Phosphorylated p44/42 MAPK

We measured the phosphorylation of Raf-1 and p42/44 MAPKs in cells incubated for 24 h with 1 μM progesterone. Total proteins were extracted and equivalent amounts of protein (60 μg) were separated by SDS-PAGE. These blots were probed with antibodies against phosphorylated (Tyr 340−341) and total Raf-1, and phosphorylated and total p42/44 MAPKs (Fig. 4A and B). Cells incubated with progesterone had less phosphorylated p42/44 MAPKs and Raf-1 than controls, but the total amounts of p44/42 MAPK and c-Raf-1 were unaltered. Since the phosphorylation of p42/44 MAPK in mast cells is inhibited by glucocorticoids via an increase in MAPK phosphatase 1 (MKP-1) [Kassel et al., 2001], we also investigated the effect of progesterone on this pathway. Progesterone did not increase the amount of MKP-1 protein (Fig. 4C).

Influence of Progesterone on CHK mRNA and Protein

Csk homologous kinase (CHK) is a tyrosine kinase that inhibits Src-family kinases by phosphorylating them. Cells incubated with 1 μM progesterone had more CHK mRNA and protein than did controls. The PCR gels shows that a characteristic 216 bp PCR fragment was more intensely stained in progesterone-treated cells than in controls (Fig. 5A). Immunoblots revealed a 52−57 kDa doublet band for CHK [Chow et al., 1994]. The bands were more

TABLE II. Percentages of Cells in the Various Phases of Cell Cycle

	Percentages of cells (%)					
	Control without progesterone			Progesterone 1 μM		
	G0/G1	S	G2/M	G0/G1	S	G2/M
Experiment 1	37	33.2	29.6	49	24.5	26.6
Experiment 2	41.8	40.6	17.6	71.4	19.4	9.2
Experiment 3	47	29	24.3	61	24.7	14.3
Experiment 4	26.3	42.7	31	40.1	39	20.9
Mean ± S.E.M.	38 ± 4.4	37.4 ± 3.7	25.6 ± 3.0	55.4 ± 6.8	26.9 ± 4.2	17.8 ± 3.8
P value (vs the absence of progesterone)				[a]*P* = 0.024	[a]ns	[a]*P* = 0.018

[a]Paired *t*-test two-tailed *P*-value.

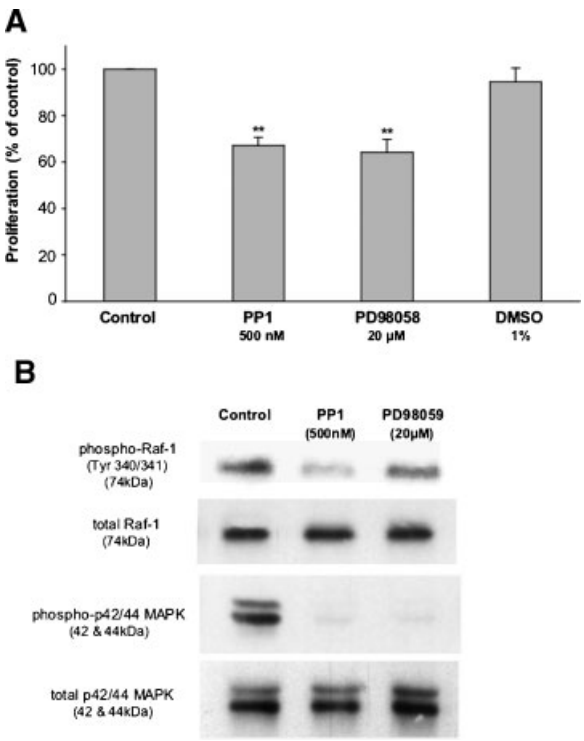

Fig. 3. Effects of PD98059 and PP1 inhibitors on HMC-1^{560} cell proliferation and Raf/MAPK signaling. **A**: Mast cells were incubated with PD98059 (20 μM), a MEK inhibitor, with PP1 (500 nM), a Src kinase inhibitor, or vehicle (DMSO 1%) for 24 h. Controls were incubated in MEM plus 10% FCS. Proliferation was measured by [^{3}H]-thymidine incorporation, and expressed as a percentage of control. Graphs show data from five independent experiments. PD98059 and PP1 reduced HMC-1^{560} proliferation, while vehicle had no effect. $^{**}P < 0.01$, n = 5, ANOVA for repeated measures followed by Tukey's multiple comparison test. **B**: Cells were incubated for 24 h with 20 μM PD98059, or 500 nM PP1. Total proteins were extracted and equivalent amounts of protein (60 μg) were separated by SDS-PAGE and blotted. Blots were probed with antibodies against phosphorylated and total Raf-1 and against phosphorylated and total p42/44 MAPKs. Representative immunoblots of three independent cultures are shown. Cells treated with PP1 had less phosphorylated forms of Raf-1 and p42/44 MAPKs than controls. In contrast, cells treated with PD98059 only had reduced amounts of phosphorylated p42/44 MAPK, while phosphorylated Raf-1 was not changed.

intense in progesterone-treated cells than in controls, although the amounts of actin were similar (Fig. 5B). The amounts of CHK in progesterone-treated cells and untreated cells

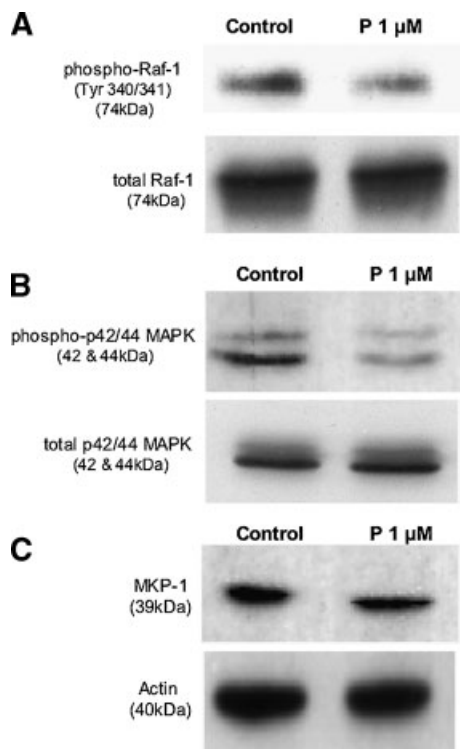

Fig. 4. Effect of progesterone on Raf-1 and p42/44 MAPKs phosphorylation, and on MAPK phosphatase 1 (MKP-1) in HMC-1^{560} cells. Cells were incubated for 24 h with or without 1 μM progesterone (P 1 μM). Total proteins were extracted and equivalent amounts of protein (60 μg) were separated by SDS-PAGE and blotted. Representative immunoblots of three independent cultures are shown. **A**: Blots were probed with antibodies against phosphorylated and total Raf-1, **B**: Blots were probed with antibodies against phosphorylated and total p42/44 MAPKs, **C**: Blots were probed with antibodies against MKP-1 and actin. There were less phosphorylated forms of p42/44 MAPKs and Raf-1 in treated cells than in controls, while the amounts of total p42/44 and Raf were similar. Progesterone did not increase the amount of MKP-1.

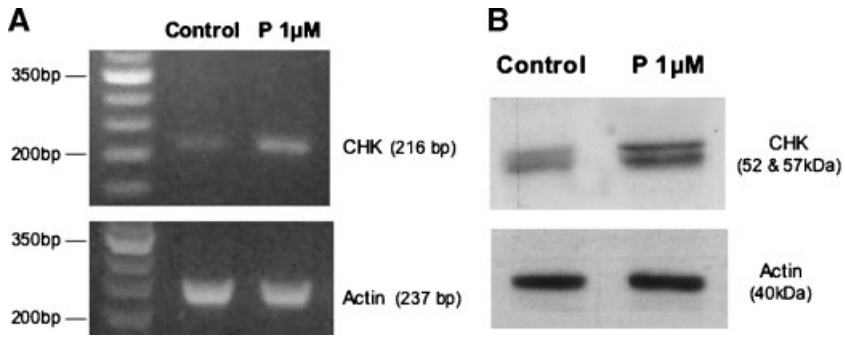

Fig. 5. Effect of 1 μM progesterone on the amounts of CHK mRNA and protein in HMC-1^{560}. **A**: The gel shows a 216 bp PCR product with the characteristic of CHK transcripts whose intensity was increased in progesterone-treated cells (one experiment of four). **B**: Blots were probed with antibodies against CHK and blots probed with antibody against actin were used as control; the intensity of the bands was measured by scan analysis software. The CHK signal was more intense in progesterone-treated cells than in untreated cells (1 representative experiment of four).

were significantly different ($^*P < 0.05$, $n = 4$, Table III).

HMC-1^{560} cells transfected with siRNA CHK had less (3.8-fold) CHK than cells transfected with non-targeting siRNA (negative controls) (Fig. 6A). Incubation with 1 μM progesterone for 24 h had no effect on the proliferation of cells transfected with siRNA CHK (Fig. 6B) but it reduced the proliferation of cells transfected with non-targeting siRNA.

DISCUSSION

Steroid hormones influence the function of mast cells and their distribution in several tissues [Beagley and Gockel, 2003]. The expansion of mast cells in vivo is inhibited by glucocorticoids [Tchekneva and Serafin, 1994]. In contrast oestradiol increases the number of mast cells in the ovarian complex of mice [Jaiswal and Krishna, 1996], and the distribution and activation of mast cells in the human endometrium are modulated throughout the menstrual cycle [Jeziorska et al., 1995]. But the specific role of progesterone in modulating the proliferation of mast cells remains unclear.

We find that progesterone inhibits the spontaneous proliferation of HMC-1^{560} mast cells, so that they proliferate only half as fast as cells cultured without hormone. Cells were arrested in the G0/G1 stage of the cell cycle with concurrent reductions in the cells in the G2/M phases. This is in agreement with a report showing that progesterone markedly inhibited the growth of PR-transfected MDA-MB-231 tumor cells by arresting cells in the G0/G1 phase of the cell cycle [Lin et al., 1999].

We have demonstrated the persistent activation of the downstream Raf/ERK pathway in HMC-1^{560} cells under our basal experimental conditions, while PD98059, a specific MEK inhibitor, reduced cell proliferation. This indicates

TABLE III. Action of Progesterone on the Amount of CHK (protein)

	Relative intensity of Western blot signal (%)			
	Actin		CHK	
	Control without progesterone	Progesterone 1 μM	Control without progesterone	Progesterone 1 μM
Experiment 1	100	75.2	100	244
Experiment 2	100	109.1	100	179
Experiment 3	100	137.8	100	414
Experiment 4	100	111	100	302
Mean ± S.E.M	100 ± 0	108.3 ± 12.8	100 ± 0	284.8 ± 49.9
P value (vs the absence of progesterone)		ns		[a]$P = 0.034$

[a]Paired t-test two-tailed P-value.

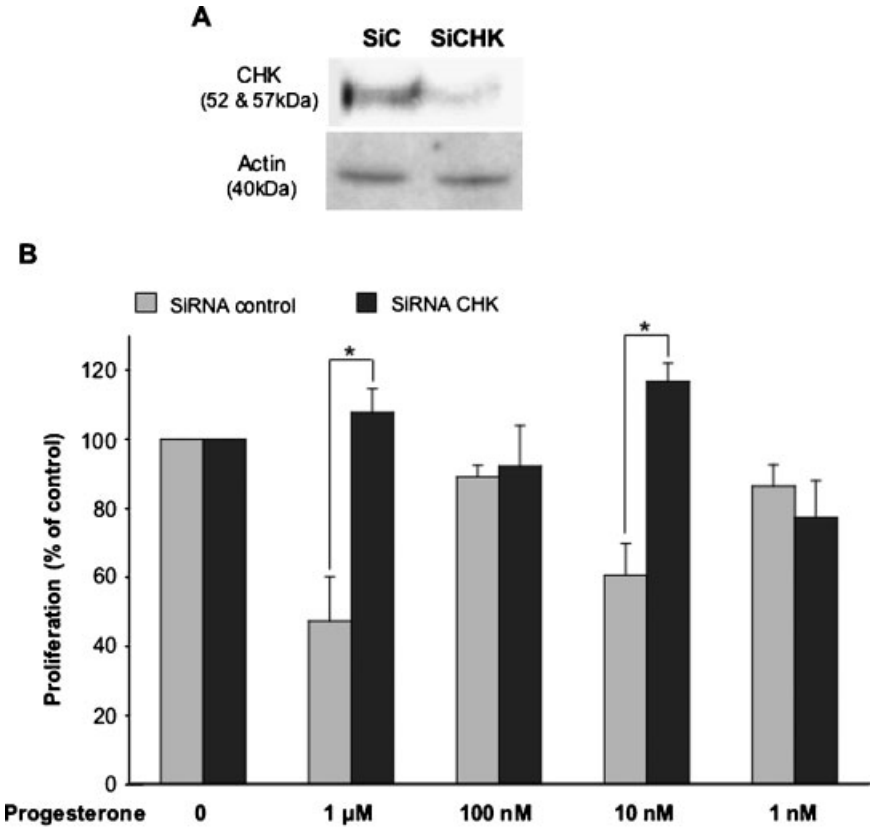

Fig. 6. Effect of progesterone on proliferation of HMC-1^{560} cells transfected with siRNA CHK. HMC-1^{560} cells were transfected with siRNA CHK using the Amaxa system. **A**: The intensities of the CHK signal in Western blots of cells transfected with siRNA CHK and with siRNA (controls) showed that there were about four-times less CHK in cells transfected with siRNA CHK than in cells transfected with non-targeting siRNA. **B**: Transfected cells were incubated with 1 µM, 100 nM, 10 nM or 1 nM progesterone for 24 h. Results are expressed as means ± SEM of triplicates of one representative experiment. Progesterone (1 µM and 10 nM) reduced the proliferation of cells transfected with non-targeting siRNA (negative control) but had no effect on the proliferation of cells transfected with siRNA CHK (Unpaired *t*-test two-tailed *P* value, $*P < 0.05$).

that HMC-1^{560} cell proliferation is closely associated with the phosphorylation of p42/44 MAPK. We also find a significant reduction in phospho-p42/44 MAPK in progesterone-treated HMC-1^{560} cells. As MAP kinases are involved in the proliferation of HMC-1 cells, any alteration in p42/44 MAP kinase activation may contribute significantly to growth arrest, and the inhibition of the p42/44 MAPK pathway may be part of the mechanism by which progesterone inhibits growth. The anti-proliferative property of progesterone is also associated with a parallel decrease in phospho-p42/44 MAPK in mammary tumor cells. The activity of the glucocorticoid receptor somewhat overlaps that of the PR in these cells [Leo et al., 2004]. A recent study also reported that glucocorticoids decrease the amounts of the phosphorylated forms of p42/44 MAPK in mast cells by increasing MAPK phosphatase 1 (MKP-1) [Kassel et al., 2001]. In contrast, we find that there is no increase in MKP-1 protein in HMC-1^{560} cells incubated with progesterone. This suggests that progesterone does not have any glucocorticoid-like effects in HMC-1^{560} cells.

Raf-1 is a key mediator of mitogen-activated growth responses. It is recruited by Ras to perpetuate mitogen responses via the mitogen-activated protein kinase pathway [Avruch et al., 2001; Marshall, 1995]. Among the proteins that modulate Raf signaling, c-src is a co-transducer of mitogenic signals emanating from tyrosine kinase polypeptide growth factor receptors. The recruitment of Src family kinases (SFKs) at specific tyrosine residues activate the Ras/Raf/Erk MAPK pathway, which plays a role in the control of mast cell proliferation [Linnekin et al., 1997; Voytyuk et al., 2003]. Further phosphorylation of ERK depends on the activity of the Src family kinases [Voytyuk et al., 2003],

and differences in the activation of the ERK pathway can be accounted for by the differential regulation of these kinases [Lennartsson et al., 1999; Bondzi et al., 2000]. Csk homologous kinase (CHK) is recruited to the plasma membrane where it phosphorylates the inhibitory C-terminal tyrosine of several Src-family kinases, including c-Src, and inactivates them [Price et al., 1999]. HMC-1^{560} mast cells incubated with the specific Src kinase inhibitor, PP1, proliferate more slowly than controls and have less phosphorylated Raf-1 and less p42/44 phosphorylated MAPK. We find that cells incubated with progesterone lose phosphorylated Raf-1. The inhibition of Raf-1 phosphorylation by progesterone probably leads to impaired MEK activity and a decrease in phosphorylated p42/44 MAPK. We also find that CHK production is significantly greater in progesterone-treated cells than in untreated cells. Other experiments showed that siRNA CHK reduces CHK synthesis in HMC-1^{560} cells and also reduces the inhibition of cell proliferation by progesterone. Thus progesterone increases CHK that reduces the proliferation of HMC-1^{560} cell proliferation. This effect parallels a loss of the active phosphorylated forms of Raf-1 and p42/44 that are implicated in the control of cell proliferation, and whose phosphorylation depends on Src kinase activity. We have yet to determine whether the increase in CHK is the cause of the progesterone-induced reduction in the amounts of active phosphorylated Raf-1 and p42/44. Several reports indicate that mutations in the juxta-membrane domain of c-kit causes constitutive activation of the c-kit receptor leading to spontaneous proliferation [Butterfield et al., 1988; Sundstrom et al., 2003]. There is no direct evidence that the inhibition HMC-1^{560} cell proliferation by progesterone has anything to do with c-kit. The protein, c-kit, belongs to the tyrosine kinase receptor family, and its activity is controlled by endogenous inhibitors like CHK. Perhaps an increase in CHK decreases c-kit activity and hence the amounts of phosphorylated Raf-1 and p42/44 MAPK, as these are commonly associated with c-kit activation and cell proliferation. Further experiments are needed to clarify this.

Progesterone was active at a concentration of 1 μM in our model system. This concentration is usually considered to be supra-physiological, although such high concentrations have been reported in the sera of certain pregnant women [Johansson and Jonasson, 1971], and have been widely used in in vitro studies. Competitive binding assays have also documented the absence of high affinity binding sites for progesterone in human immune cells [Neifeld et al., 1977], but this remains to be verified. One recent report highlighted the non-transcriptional activity of progesterone; the increase in the intracellular concentrations of cAMP and cGMP was used to explain the anti-proliferative effect of progestins [Sager et al., 2003]. Again, further experiments are needed to determine how progesterone up-regulates CHK.

In conclusion, our findings provide new insights into the way progesterone inhibits cell proliferation by highlighting its effect on CHK, a Src kinase inhibitor. In view of the complex functions of progesterone, identifying a new pathway by which the steroid inhibits proliferation might open the way to new strategies for designing therapeutic agents.

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