Identification of Extracellular Signal-regulated Kinase 1/2 and p38 MAPK as Regulators of Human Sperm Motility and Acrosome Reaction and as Predictors of Poor Spermatozoan Quality

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Mature spermatozoa acquire progressive motility only after ejaculation. Their journey in the female reproductive tract also includes suppression of progressive motility, reactivation, capacitation, and hyperactivation of motility (whiplash), the mechanisms of which are obscure. MAPKs are key regulatory enzymes in cell signaling, participating in diverse cellular functions such as growth, differentiation, stress, and apoptosis. Here we report that ERK1/2 and p38 MAPK are primarily localized to the tail of mature human spermatozoa. Surprisingly, c-Jun N-terminal kinase 1/2, which is thought to be ubiquitously expressed, could not be detected in mature human spermatozoa. ERK1/2 stimulation is downstream to protein kinase C (PKC) activation, which is also present in the human sperm tail (PKCβ1 and PKCε). ERK1/2 stimulates and p38 inhibits forward and hyperactivated motility, respectively. Both ERK1/2 and p38 MAPK are involved in the acrosome reaction. Using a proteomic approach, we identified ARHGAP6, a RhoGAP, as an ERK substrate in PMA-stimulated human spermatozoa. Inverse correlation was obtained between the relative expression level of ERK1 or the relative activation level of p38 and sperm motility, forward progression motility, sperm morphology, and viability. Therefore, increased expression of ERK1 and activated p38 can predict poor human sperm quality.

Sperm are immotile in the testes and they acquire progressive motility after ejaculation. They cross the uterine cervix, undergo suppression of progressive motility, become capacitated (capable to fertilize), including resumption of another form of motility, hyperactivation, bind the egg, undergo the acrosome reaction, and fertilize it (1, 2). Relatively little is known about the molecular mechanisms that mediate spermatozoan functions in general and the various forms of motility in particular (1, 2).

MAPK3 cascades play a crucial role in metazoan development, including fate determination, differentiation, proliferation, survival, migration, growth, cell cycle progression, and apoptosis (3–7). MAPK cascades consist of several tiers of protein kinases that activate each other by sequential phosphorylation. Four major MAPK cascades are known in mammals: ERK1–2, JNK 1–3, p38 α–δ, and ERK5 (big MAPK; BMK) (3–7). Activation of MAPK is initiated by activation of MAP3Ks by small G-proteins of the Ras family (e.g. Ras, CDC42, and Rac), followed by sequential activation of MAPKs and MAPKs. The prototypic ERK cascade is activated by growth factors, mitogens, and G-protein-coupled receptors (GPCR) and consists of Rafs (MAP3K), MEK1/2, ERK1/2, and several MAP-activating protein kinases (MAPKAPKs). The stress-activated protein kinases are now known as JNK and p38 MAPK. Both are initiated by stress stimuli, GPCRs, inflammatory cytokines, and growth factors. JNK1–3 consists of a sequential activation of Rac1/Cdc42, mixed lineage kinases as MAP3Ks, MAP kinases 4 and 7, and JNK1–3. The p38 MAPK cascade is composed of sequential activation of MAP3Ks, MKK3/4/6, p38α–δ, and several MAPKAPKs (3–7).

Mature spermatozoa are fully differentiated cells that lack active transcriptional machinery. Hence, it is of great interest to

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¶ This abbreviation used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; OAG, 1-oleoyl-2-acetylglycerol; JNK, c-Jun N-terminal kinase; GPCR, G-protein-coupled receptor; MBP, myelin basic protein; CASA, computer-aided sperm analysis; SM, sperm motility; FPM, forward progression motility; ROC, receiver operating characteristic; AUC, area under the curve; BAPTA/AM, 2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester); PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; BSA, bovine serum albumin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PMA, phorbol myristate acetate.
investigate whether ejaculated human spermatozoa express and utilize MAPKs, which specialize in growth, differentiation, and stress. Because spermatozoa lack those functions, the studies might point to novel functions mediated by MAPKs. During spermatogenesis, MAPKs mediate cell division, differentiation, survival, adhesion, and death (8). MAPKs were reported to play a role during meiotic progression of mouse spermatocytes (9–12) and in adhesion function at the Sertoli-germ cell interface (8). On the other hand, the role of MAPK in mature mammalian spermatozoan motility, capacitation, and acrosome reaction is limited and controversial as detailed below (10, 13–17). In particular the role of JNK and p38 MAPK in ejaculated spermatozoa is not known (8). We therefore inquired whether ejaculated mature human spermatozoa express and utilize the various MAPKs (ERK, JNK, and p38) (6, 7), which may orchestrate the fine-tuning of sperm motility. Our results indicate that ERK1/2 and p38 MAPK, but not JNK1/2, are expressed in the tail of ejaculated human spermatozoa. Activation of ERK1/2 is downstream to PKC activation, and two of its isoforms (PKCβI and PKCε) are also present in the human sperm tail (18). Surprisingly, we discovered that ERK stimulates and p38 MAPK inhibits forward and hyperactivated motility, respectively. We also found that both ERK1/2 and p38 MAPK are positively involved in PKC-mediated acrosome reaction. Using a proteomic approach, we identified ARHGAP6, a RhoGAP, as an ERK substrate in PMA-stimulated human spermatozoa. Finally, we found that inverse correlation exists between ERK1 and phospho-p38 and sperm motility, forward progression motility, sperm morphology, and viability. Indeed, increased expression of total ERK1 and activated phospho-p38 MAPK could predict poor human sperm quality.

**EXPERIMENTAL PROCEDURES**

**Preparation of Human Spermatozoa**—Human semen was obtained from healthy donors with normal sperm density, motility, and morphology according to World Health Organization guidelines (19) or from males attending either the Male Infertility Unit, Assaf Harofeh Medical Center, Israel, or the Andrology Laboratory in Assuta Hashalom Medical Center, Tel Aviv, Israel. The human semen was liquefied for 60 min at 36 °C. Sperm was washed twice with Ham’s F-10 medium containing bovine serum albumin (BSA, 0.3%) and incubated with the medium for 3 h for capacitation (20). PMA or progesterone (Sigma) were added with or without the PKC inhibitor GF109203x (Calbiochem) or the MEK1/2 inhibitors PD98059 and U0126, or the p38 inhibitors SB203580 and PD169316 (Biomol), or the Ca2+ inhibitors EGTA, nifedipine, and BAPTA/AM (Sigma) to the above medium as described in the legends.

**Immunocytochemistry**—Human sperm (1.5 × 106) were collected on glass slides by cytospin (600 rpm). The cells were fixed and permeabilized by cold methanol (10 min), followed by cold acetone (10 min) (20). The cells were treated with the respective anti-general MAPK (ERK, JNK, and p38) antibodies (Sigma) or antibodies against mSos, MEK1/2, Raf-1, or tubulin (Santa Cruz Biotechnology) (1:25) for 18 h at 4 °C. Cells were then treated with biotinylated secondary antibody (1:100) for 30 min. After washing in PBS the cells were further treated for 30 min at room temperature using the avidin-biotin complex method as described previously (20). Visualization was performed in solutions containing diaminobenzidine (0.25 mg/ml) and 0.01% H2O2 in PBS. Specificity of the MAPK staining was confirmed by omission of the primary antibody and by the preabsorption of the antibody with an excess of the respective MAPK peptide (Sigma). All the reagents for the immunohistochemistry studies were obtained from Vector Laboratories (Burlingame, CA).

**Immunoelectron Microscopy**—Human sperm were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer for 60 min at room temperature (21). After washing, the tissue samples were dehydrated in acetone and embedded in araldite. The cells were not treated with osmium because of loss of antigenicity in osmium-treated cells. Sections were placed on silver grids, and immunogold labeling was performed as described previously (21). Sections were treated with 0.1% Triton X-100 in PBS for 20 min, washed in PBS, and placed in 1% BSA for 1 h. After draining, the sections were incubated with anti-ERK antibody (1:50) (Sigma) for 18 h at 4 °C. After rinsing in 0.05 M Tris-buffered saline (TBS), pH 7.3, the sections were incubated for 1 h with 8- or 15-nm gold-conjugated goat anti-rabbit IgG (Biocell, Cardiff, UK) and diluted 1:10 with TBS, pH 8.4, containing 1% egg albumin. Following the immunostaining, sections were rinsed, and contrast was enhanced with uranyl acetate and lead citrate. Sections were then examined with a JEOL 100B electron microscope.

**Indirect Immunofluorescence**—Sperm were washed and smeared on polylysine slides and then allowed to air-dry. Cells were washed with PBS, permeabilized with Triton X-100, 0.5% buffered in PBS. Nonspecific binding was blocked with 3% BSA buffered in PBS. Cells were probed first with monoclonal anti-p38 (1:100) or anti-ERK1/2 (1:100) polyclonal antibody, washed three times with PBS, and then probed with a secondary anti-rabbit Hileyte FluorTM 488-labeled antibody or a secondary anti-mouse Hileyte FluorTM 647-labeled antibody (Anaspec). Slides were viewed with a laser scanning microscope (510, Zeiss).

**Activation of MAPK Cascades**—Capacitated and noncapacitated human spermatozoa were prepared as above and stimulated with PMA, progesterone, or other drugs as indicated (Sigma), and cell extract was used for Western blotting. After stimulation, the cells were diluted immediately with excess Ham’s F-10 medium, precipitated by centrifugation at 15,000 × g for 20 s at room temperature, and washed once more, and the resulting pellets were stored at −20 °C until used. The thawed pellets (all these steps were made at 4 °C) were resuspended in a minimal volume of lysis buffer (50 μl per 3 × 107 cells) made of 50 mm Tris-HCl, pH 8.0, 2 mm EGTA, 20 mm NaCl, 1.0 mm sodium orthovanadate, 25 mm β-glycerophosphate, 100 nm okadaic acid, 0.50% Nonidet P-40, 1 mm benzamidine, 10 μg/ml aprotonin, 10 μg/ml leupeptin, 1 mm phenylmethylsulfonyl fluoride, and 2 mm dithiothreitol. The suspensions tubes were incubated on ice for 10 min and centrifuged (15,000 × g, 15 min, 4 °C). The supernatants, which contained unbound proteins, were collected, and aliquots from each sample (20 μg) were separated on 10% SDS-PAGE followed by Western blotting with mouse monoclonal anti-active (doubly phosphorylated) MAPKs (ERK, JNK, and p38) (Sigma), MPM2 (anti-phos-
**ERK and p38 Are Regulators of Sperm Motility**

**FIGURE 1.** A, localization of MAPK in ejaculated human spermatozoa. Human spermatozoa were immunostained with antibodies to ERK (panels a and b), Raf-1 (panel c), MEK1 (panel d), p38 (panels e and f), JNK (not shown), and tubulin (panel g). For ERK and p38, but not JNK, immunostaining can be observed in the sperm neck and along the entire tail. Preabsorption of the antibodies with the relevant peptide antigen resulted in disappearance of the staining (panels b and f). The staining of mSos (not shown), Raf-1 (panel c), and MEK1 (panel d) is similar to that of ERK, with heavy staining of the neck and along the tail. Note the lack of tubulin immunostaining in the neck (panel g). Cells shown are representative of at least four independent experiments. B, localization of ERK in human spermatozoa by immunogold labeling. An anti-ERK polyclonal antibody and goat anti-rabbit gold-labeled particles (8 and 15 nm) were used on araldite-embedded thin sections of glutaraldehyde-fixed (unsmicated) human spermatozoa. Panel a, localization of ERK in cross-section of the principal piece. Gold particles are distributed in the outer dense fibers. Panel b, lack of staining with the peptide-preabsorbed antibody. Cells shown are representative of three independent experiments.

**FIGURE 2.** Fluorescence microscopy for expression of ERK1/2 and p38 in human spermatozoa. Precapacitated spermatozoa were reacted with anti-ERK (A) and anti-p38 (B) antibodies. Phase-contrast images are shown for ERK1/2 and p38 immunolabeling in B and F, respectively, and negative controls with secondary antibodies are shown in C and G. Phase-contrast images are shown for controls with secondary antibodies in D and H. Like the diaminobenzidine staining of Fig. 1, ERK1/2 was distributed along the tail, whereas p38 seems to localize also in post-acrosomal regions, the mid-piece, and the tail (C). Scale bars indicate 10 μm.

Phosphorylation of MAPKs was detected with polyclonal antibodies for the various general MAPKs (Sigma) as a control. The blots were developed with alkaline phosphatase- or horseradish peroxidase-conjugated anti-mouse or anti-rabbit Fab antibodies (Jackson ImmunoResearch). The blots were autoradiographed on Kodak X-100 films, and the phosphorylation was quantified by densitometry (690 densitometer, Bio-Rad). Each band from the anti-phospho-MAPK (ERK, JNK, and p38) was normalized to the corresponding band from the anti-general MAPK antibodies blot for even loading (22).

**Recombinant Expression of pERK**—To obtain doubly phosphorylated ERK, activated human GST-ERK2 was co-expressed with constitutively active MEK1 in BL21 bacteria, as described (23). The bacteria were grown in 2YT medium at 30 °C to an absorbance of 0.6, and then 1 mM isopropyl-1-thio-β-D-galactopyranoside was added for an additional 4 h. Proteins were purified over glutathione-Sepharose 4B (Amersham Biosciences) or nickel-NTA-agarose (Qiagen) according to the manufacturer’s instructions.

**Cell Culture, Transfection, and Immunoprecipitation**—Human embryonic kidney 293 cells (HEK293) were grown in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum in 95% air, 5% CO2 at 37 °C. Cells were plated in 100-mm plates 24 h prior to transfection and were transfected with 4 μg of plasmid DNA ARHGAP6-GFP, using the calcium phosphate method, and grown as above. 16 h post-transfection, the medium was replaced, and 48 h later the cells were lysed. Lysis buffer contained 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.5 mM EDTA, 15% glycerol, 0.2% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors mixture. The lysate was subjected to immunoprecipitation with an anti-GFP antibody (Roche Applied Science) conjugated to protein A/G PLUS-agarose (Santa Cruz Biotechnology) by gentle shaking for 3–4 h in 4 °C, and washed three times with lysis buffer. Samples were frozen in −20 °C and were later subjected to immunoprecipitation. Where appropriate, immunoprecipitated ARHGAP6 was attached to beads (15 μl) (0.5 μg per reaction) was mixed with immunoprecipitated ERK attached to beads (15 μl). The buffer
ERK and p38 Are Regulators of Sperm Motility

...reaction mix (3×) (75 mM β-glycerophosphate, 1.5 mM dithiothreitol, 3.8 mM EGTA, 0.15 mM orthovanadate, 30 mM MgCl₂, 30 μM calmodizolium, 0.3 mM ATP), containing 100 μM [γ-32P]ATP (4000 cpm/pmol), was added to the reaction in a final volume of 30 μl and incubated for 20 min at 30°C. The reaction was terminated by adding 10 μl of 4× Sample buffer, and the phosphorylated proteins were resolved on SDS-PAGE. Phosphorylation of MBP (8 μg per reaction) by ERK was carried out as a positive control.

Assessment of Sperm Motility—Progressive flagellar motility was determined manually (20) or by using Computer-aided Sperm Analysis (CASA) (Sperm Analysis System version 12-IVOS, Hamilton Thorne Biosciences, Beverly, MA), which was also used to measure hyperactivation (24). Hyperactivation parameters were as follows: curvilinear velocity >100 μm/s, linearity <60%, amplitude of lateral head >5 μm.

Assessment of Sperm Acrosome Reaction—The percentage of acrosome-reacted sperm was determined microscopically on air-dried sperm smears using FITC-conjugated Pisum sativum agglutinin, which is a fluorescent lectin capable of binding to the acrosomal content. Washed cells (10⁶ cells/ml) were capacitated for 3 h at 37°C and 0.5% CO₂ in F-10 medium supplemented with BSA 3 mg/ml (20). The inhibitors were added for the last 10 min of incubation, and then PMA (100 nM) was added for another hour. At the end of incubation an aliquot of the sperm was spread on microscope slides and allowed to air-dry. The sperm were then permeabilized by methanol for 15 min at room temperature, washed once with 25 mM Tris-buffered saline, pH 7.6, for 5 min and twice with H₂O at 5-min intervals, air-dried, and then incubated with FITC-P. sativum agglutinin (60 μg/ml) for 1 h, washed twice with H₂O at 5-min intervals, and mounted with FluoroGuard Antifade (Bio-Rad). For each experiment, at least 150 cells per slide in duplicates were evaluated. Cells with green staining over the acrosomal cap were considered acrosome-intact; those with equatorial green staining or no staining were considered acrosome-reacted. The analysis of multiple groups was performed by one-way analysis of variance using SPSS software with p < 0.05 considered significant.

Correlation between MAPK Levels and Sperm Quality Parameters—Human spermatozoa from 47 males attending the Male Infertility Unit were analyzed for MAPK levels and activity as above and correlated with percent sperm motility (SM), forward progression motility (FPM), morphology, and viability (19). The correlations were obtained using Pearson correlation coefficients. Logarithmic transformation (when needed) was used to obtain normal distribution. Aiming to discriminate normal and abnormal motility and FPM samples, single variable analysis was used utilizing Student’s t test. Stepwise logistic regression was used for multivariate analysis. p value <0.05 was considered statistically significant. To define optimal cut points for MAPK levels, receiver operating characteristic (ROC) analysis (25) was used discriminating normal versus abnormal motility and FPM. We calculated the area under the curve (AUC), which represented the accuracy of the test, and a p value <0.05 was considered predictive.

Data Analysis—Results from two or three experiments were expressed as mean ± S.D. Data were subjected to statistical analysis with one-way analysis of variance and Fisher’s post-hoc analysis with one-way analysis of variance and Fisher’s protected least significant difference tests, and statistical significance was accepted when p < 0.05. Other statistical tests are detailed in the legends.

RESULTS

ERK1/2 (and its cascade members Sos/Raf-1/MEK1) and p38 MAPK, but not JNK, were identified in the tail of mature ejaculated human spermatozoa by immunocytochemistry (Fig. 1A). The ERK cascade (Sos/Raf-1/MEK1/ERK1/2) and p38 were localized in the neck and distributed along the mid-, principal, and end pieces of the tail. Tubulin in the axoneme was evenly distributed along the tail. Electron microscopy revealed (Fig. 1B) that ERK1/2 was localized to the outer dense fibers. Confocal microscopy revealed that ERK1/2 is distributed mainly to the entire mid-piece (Fig. 2), whereas p38 is primarily localized to the upper mid-piece (Fig. 2).

We then identified the presence of active ERK1/2 and p38 in human ejaculated spermatozoa by Western blotting and com-
pared it with the known PMA-stimulated MAPK in the mouse pituitary gonadotrope L/H9252T2 cells (22) (Fig. 3). Human ejaculate spermatozoa express mainly ERK2, which was stimulated by PMA or by the general activator vanadate peroxide (Na3VO4). PMA serves as an analog of diacylglycerol, the physiological activator of PKC, and both stimulants revealed the activation of ERK1. The sperm-ligand progesterone, reported to activate human sperm ERK (26), had no effect. p38 MAPK in its phosphorylated form was very low in control L/H9252T2 cells but was present in spermatozoa. We could not detect further activation of phospho-p38 by PMA, vanadate, nor progesterone versus a positive effect of PMA in L/H9252T2 cells (Fig. 3). The data suggest that ejaculate sperm p38 MAPK is fully phosphorylated, hence activated. p38 MAPK is encoded by four genes that yield nine isoforms (5), and we could detect mainly p38α and occasionally p38β. JNK1/2 is present in L/H9252T2 cells and is activated by PMA but is undetectable in PMA-, vanadate-, or progesterone-treated spermatozoa.

We then incubated spermatozoa for 2.5 h in a capacitation medium (Fig. 4) to reach capacitation in vitro (20). Exposure to PMA (15 min) throughout the entire capacitation process further stimulated ERK1/2 levels in comparison with controls (*, p < 0.05; **, p < 0.01). Thus, ERK activation by PMA can be detected in both noncapacitated and capacitated spermatozoa. ERK2 activation by PMA was sensitive to the MEK1/2 inhibitors PD98059 and U0126 (Fig. 5A, left panel), confirming the functioning of a canonical MEK1/2-ERK1/2 cascade (6, 7) in spermatozoa and the use of the two drugs in the studies described below. The selective p38 inhibitor SB203580 had no effect on PMA to ERK signaling, ruling out an inhibitory cross-talk between p38 MAPK and ERK (27). The selective pan-PKC inhibitor, GF109203X, completely abolished the PMA-induced activation of ERK1/2, indicating that the PMA effect was PKC-dependent (Fig. 5A, right panel). ERK activation by PMA was insensitive to EGTA (extracellular Ca2+ chelator), to nifedipine (voltage-dependent Ca2+ channel blocker), or to BAPTA/AM (intracellular Ca2+ chelator) (Fig. 5B), unlike L/BT2 cells, in

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**FIGURE 4.** Activation of ERK1/2 by PMA in human spermatozoa. Human sperm were incubated in capacitation medium. At the end of each preincubation time, cells were treated with PMA (100 nM) for 15 min. Cell lysates were subsequently prepared, ERK1/2 activity (upper and lower graphs, respectively) was determined as in Fig. 3. A representative blot is shown, and bars are mean ± S.D. from three experiments. *, p < 0.05; **, p < 0.01.

**FIGURE 5.** A, PMA-induced ERK activation in human spermatozoa is sensitive to MEK1/2 inhibitors and insensitive to p38 inhibitor (left panel) and to the pan-PKC inhibitor GF109203X (right panel). Capacitated human sperm were preincubated with the specific MEK1/2 inhibitors PD98059 (50 μM) and U0126 (50 μM), the p38 inhibitor SB203580 (50 μM) and the PKC inhibitor GF109203X (3 μM) for 15 min and PMA (100 nM) was added for another 15 min. Cell lysates were subsequently prepared and ERK2 activity was determined as in Fig. 3. Bars are mean ± S.D. from three experiments. Means designated by different letters are significantly different (p < 0.05).

B, PMA-induced ERK activation is Ca2+-independent. Capacitated human spermatozoa were preincubated with or without various Ca2+ blockers: nifedipine (L-type voltage-dependent Ca2+ channel blocker, 1 μM), EGTA (a general Ca2+ chelator, 5 mM) and BAPTA/AM (intracellular Ca2+ chelator, 50 μM) for 30 min. PMA (100 nM) was then added for 15 min and ERK2 activity was determined as in Fig. 3. A representative blot is shown and bars are mean ± S.D. from three experiments. Means designated by different letters are significantly different (p < 0.05).
which activation of ERK by PMA was markedly reduced by the above Ca\(^{2+}\) inhibitors (Fig. 5B).

Incubation of spermatozoa with normal motility (>50% motility) (19) with the selective MEK1/2 inhibitor PD98059 reduced flagellar motility (Fig. 6A) in comparison with control (*, \(p < 0.05\); **, \(p < 0.01\)). In contrast, incubation of spermatozoa with low motility (~30% motility) (19) with the p38 inhibitor SB203580, significantly stimulated flagellar motility (Fig. 6A). Similarly, we could significantly increase sperm forward motility, even for spermatozoa with very low motility (~30% motility) by incubation with PMA, or the p38 inhibitor SB203580, or another selective p38 inhibitor PD169316 for 15 min, with no additive effect with PMA (Fig. 6B). Similar results were found with sperm of normal motility. We also examined forward and hyperactivated motility using CASA (Fig. 6, C and D, respectively). Studies have indicated that the direct quantitative assessment of sperm motility by CASA accurately reflects the fertilizing ability of human spermatozoa in vitro in particular where the conventional semen analysis is of limited diagnostic value (28, 29). PMA increased forward (Fig. 6C) and hyperactivated motility (Fig. 6D) (see also supplemental video 1). The inhibitors SB203580 and PD169316. Stimulation of the acrosome reaction by PMA (20) was nearly abolished by the MAPK inhibitors. Further support comes from the observation that OAG, which activates ERK (Fig. 7A), is also capable of activating the acrosome reaction (18). Hence, despite their opposing role in motility, ERK1/2 and p38 MAPK are positively implicated in the acrosome reaction.

We then took a proteomic approach to identify ERK substrates in human spermatozoa. Control and PMA-treated cells were subjected to gel electrophoresis followed by Western blotting using phospho-specific anti-ERK (MAPK) substrates antibodies (MPM2) (Fig. 8). Human spermatozoa were incubated with PMA, a known inducer of the acrosome reaction (31–33), and the MEK1/2 inhibitors PD98059 and U0126 or the p38 inhibitors SB203580 and PD169316 (50 \(\mu M\) each) for 15 min, and PMA (100 nM) was added where indicated for another 15 min. Forward motility (C) and hyperactivation of motility (hyperactivated/total motile cells) (D) were measured using CASA. Results are mean ± S.D. from three to six experiments. Means designated by different letters are significantly different, * \(p < 0.05\); **, \(p < 0.01\).
viability (19). Among ERK1, ERK2, and p38 MAPK (phosphorylated and nonphosphorylated forms), only total ERK1 and phosphorylated p38 gave highly significant inverse correlation to all the sperm quality parameters (Table 1). Univariate analysis revealed that ERK1 and phospho-p38 are statistically significant discriminators for abnormal sperm motility ($H1102150\%$) and abnormal forward progression motility ($H110213$) (19) (Table 2). Multivariate analysis (logistic regression), which included the age of the donor, enzyme levels, and sperm quality parameters, revealed phospho-p38 to be a single and independent discriminator for abnormal sperm motility ($p < 0.001$) (19) and abnormal forward progression motility ($<3$) (19) (Table 2). Multivariate analysis (logistic regression), which included the age of the donor, enzyme levels, and sperm quality parameters, revealed phospho-p38 to be a single and independent discriminator for abnormal sperm motility ($p = 0.001$). Similarly, ERK1 is a single discriminator for forward progression motility ($p = 0.003$). Using ROC analysis, a cut point of 1.40 for log phospho-p38 (at 25.12% compared with control) could distinguish normal ($\geq50\%$) from abnormal motility with $80\%$ sensitivity and $81.5\%$ specificity; and abnormal forward progression motility ($<3$) with $84.2\%$ sensitivity and $82.1\%$ specificity; AUC is 0.880 (Table 3 and Fig 10).

**FIGURE 7. Effect of the diacylglycerol analog OAG on ERK1/2 activation (A) and forward (B) and hyperactivated (C) motility in ejaculated human spermatozoa.** A, capacitated human spermatozoa were stimulated with increasing concentrations of OAG or with PMA (100 nM) for 15 min. After treatment, cell lysates were analyzed for ERK activity by Western blot using an antibody for phospho-ERK. Total ERK was detected with polyclonal antibodies as a control for sample loading. A representative blot is shown and similar results were observed in two other experiments. B and C, capacitated human spermatozoa ($\sim40\%$ motility) were incubated with OAG (60 $\mu$g/ml) for 15 min. Forward motility ($\beta$) and hyperactivation of motility (hyperactivated/total motile cells) ($\gamma$) were measured using CASA. Results are mean $\pm$ S.D. from three experiments. Means designated by different letters are significantly different ($p < 0.05$).

**FIGURE 8. Role of MAPK in acrosome reaction.** Human sperm were preincubated in capacitation medium for 3 h. The specific MEK1/2 inhibitors PD98059 (PD) and U0126, or the p38 inhibitors SB203580 (SB) and PD169316 (50 $\mu$M, each) were added for the last 10 min of the preincubation, and PMA (100 nM) was added for another hour. The percentage of acrosome-reacted cells was determined using FITC-conjugated *P. sativum* agglutinin (PSA-FITC). The basal acrosome reaction was $21\%$. The data represent the mean $\pm$ S.D. of duplicates from 6 to 8 experiments after subtracting the basal percentage from the induced acrosome reaction. Means designated by different letters are significantly different ($p < 0.05$).

**DISCUSSION**

We first identified the Sos/Raf-1/MEK1/ERK1/2 pathway and p38 MAPK in mature ejaculated human spermatozoa by immunocytochemistry, immunofluorescence microscopy, and Western blotting. Surprisingly, we could not detect the other stress-activated MAPK, namely JNK by the above methods. We therefore postulated that JNK may be involved in spermatogenesis but not in mature spermatozoa. Indeed, we found that JNK expression is transient as it appears in hamster round spermatids, declining thereafter.4 Nevertheless, the lack of expression of JNK in ejaculated spermatozoa is interesting because JNK is thought to be ubiquitously expressed (34) and JNK1/2/3 knock-out mice are fertile (35).

We found ERK and p38 MAPK in the neck of mature human spermatozoa, and like PKCβ1 and PKCe (20), they were distributed along the mid-, principal, and end pieces of the tail. Mature human sperm express mainly ERK2 under basal conditions, and activation by PMA revealed the presence of ERK1. The p38 MAPK is apparently fully activated, because PMA, vanadate, and progesterone had no effect on p38 MAPK phosphorylation. p38 MAPK is encoded by four genes that yield nine isoforms (5), and we could detect mainly p38α.

As PMA serves as an analog of diacylglycerol, the physiological activator of PKC, we reasoned that diacylglycerol may be a sperm activator, and therefore we evaluated its ability to stimulate ERK (36). Indeed we show here that a cell-permeable analog of diacylglycerol, namely OAG, is capable of activating ERK and also mimics other functions of PMA on human spermatozoa.

4 R. Golan, L. Shochat, T. Almog, and Z. Naor, manuscript in preparation.
ERK and p38 Are Regulators of Sperm Motility

zoa such as increased motility, hyperactivation of motility, and induction of acrosome reaction.

Activation of ERK downstream to PKC as revealed by PMA stimulation in human spermatozoa differs from somatic cells in its insensitivity to both extracellular and intracellular Ca\(^{2+}\) removal. In most cells, activation of ERK by GPCRs and via PKC is Ca\(^{2+}\)-dependent (37, 38), whereas we present evidence here that activation of spermatozoan ERK by PMA is Ca\(^{2+}\)-independent.

Mammalian spermatozoa express the G-protein G\(_q\), phospholipase C\(_\alpha\), and inositol 1,4,5-trisphosphate receptors (39, 40). We found PKC\(\varepsilon\) in the tail (20), and PKC\(\varepsilon\) is implicated in ERK activation in general (41). Hence, a G\(_q\)/phospholipase C\(_\alpha\)/diacylglycerol/PKC/ERK signaling cascade seems to operate in the human sperm, apparently in parallel to the progesterone or bicarbonate/cAMP/Ca\(^{2+}\) system (42).

We also looked for the role of ERK and p38 MAPK in sperm functions. The role of ERK in sperm motility is controversial. A positive role for ERK was suggested in fowl sperm (43). ERK was shown to be gradually activated throughout the transition of mouse spermatozoa from the caput (immotile) to the vas deferens (fully motile) (10). A contradictory report concluded that ERK inhibits motility of human spermatozoa (16). We report here that PMA increased forward and hyperactivated motility in an ERK-dependent manner. Interestingly, we found that ERK and p38 MAPK

### Table 1

**Correlation between MAPK levels and sperm quality parameters**

Human semen samples from 47 males attending the Male Infertility Unit were analyzed for SM, FPM grade, morphology, and viability. MAPK levels were determined as described under “Experimental Procedures.” The correlation between MAPK levels and sperm quality parameters was carried out after logarithmic transformation by Pearson correlations. \(r\) indicates Pearson correlation coefficient.

| Viability | Morphology | Forward progression motility | Motile sperm |
|-----------|------------|----------------------------|--------------|
| ERK1      | 0.000002   | –0.630                     | 0.0001       | 0.0001 | –0.553 |
| ERK2      | 0.063      | –0.274                     | 0.006        | –0.398 | 0.026  | –0.324 |
| P38       | 0.014      | –0.355                     | 0.003        | –0.431 | 0.008  | –0.383 |
| Phos-ERK1 | 0.151      | –0.213                     | 0.186        | –0.196 | 0.17   | –0.214 |
| Phos-ERK2 | 0.211      | –0.186                     | 0.072        | –0.265 | 0.253  | –0.170 |
| Phos-p38  | 0.00005    | –0.493                     | 0.0003       | –0.568 | 0.0002 | –0.582 |

### Table 2

**Correlation between MAPK levels and sperm quality parameters**

Univariate analysis of SM (abnormal <50%) and FPM (abnormal grade <3) versus logarithmic transformation of MAPK levels were obtained using Student’s t test. NS indicates not significant.

| Forward progression motility (0–4) | % Motile sperm |
|-----------------------------------|----------------|
| p value                           | <3 (n = 19) | ≥3 (n = 28) | <50% (n = 20) | ≥50% (n = 27) |
|                                  | S.D. Mean | S.D. Mean | S.D. Mean | S.D. Mean | S.D. Mean | S.D. Mean |
| ERK1                              | 0.000007  | 0.853  | 1.151   | 0.366  | 0.093   | 0.000004 | 0.920  | 0.951  | 0.512  | 0.208  |
| ERK2                              | 0.015     | 0.221  | 2.064   | 0.157  | 1.913   | NS       | 0.243  | 2.022  | 0.153  | 1.934  |
| Phos-p38                          | 0.000003  | 0.518  | 1.631   | 0.504  | 0.960   | 0.000004 | 0.428  | 1.638  | 0.517  | 0.915  |
regulate spermatozoan forward and hyperactivated motility in an opposing fashion. ERK was found to stimulate, whereas p38 MAPK inhibits forward and hyperactivated motility. Indeed, we could double the forward and hyperactivated motility, even when it was very low (20–30% motility; normal is regarded as >50%) (19), by incubating the sperm with the p38 inhibitors SB203580 and PD169316 or PMA. Hence forward progressive motility, suppression of motility, and hyperactivation of motility (1) may be maintained by the ERK/p38 ratio.

Sperm flagellar motility requires active sliding of microtubules by the ATPase activity on the dynein arms of the outer doublet microtubules, but the sliding mechanism is not clear (44). It is therefore reasonable to assume that protein phosphorylation is involved in flagellar motility. We therefore took a proteomic approach to initiate the identification of ERK substrates in human spermatozoa. Using phospho-specific anti-MAPK substrates antibodies (MPM2) and MALDI-TOF mass spectrometry, we identified ARHGAP6, a RhoGAP, as an ERK substrate in PMA-stimulated spermatozoa. We further confirmed the identification by showing the presence of ARHGAP6 in human spermatozoa and the phosphorylation of ARHGAP6 by ERK2 in vitro. A study of ARHGAP6 functional analysis found that ARHGAP6 co-localizes with actin filaments through its N-terminal domain and recruits F-actin into growing processes (45). ARHGAP6 has two independent functions as follows: a specific GAP for RhoA, and a promoter of actin remodeling (45). As RhoA is implicated in cell motility (55, 56) and actin remodeling is implicated in hyperactivated motility and the acrosome reaction (46, 47), phosphorylation of sperm ARHGAP6 by ERK may be involved in sperm motility, capacitation, and acrosome reaction via RhoA. Other potential substrates are the dyneins, the sperm motor molecules of the axoneme (48), known to be phosphorylated during activation (49). ERK and p38 may have opposing effects on the phosphorylation of axonemal proteins such as the dyneins. Hence, ERK and p38 may be involved in sperm chemotaxis (2), as a molecular mechanism navigating the sperm to the egg. Further studies are required to examine this proposal.

Capacitation is associated with reorganization of the plasma membrane as a result of cholesterol efflux, calcium influx, tyrosine phosphorylation (50), and an increase in cAMP (51). Capacitation culminates in the process known as acrosome reaction, which is an exocytotic secretion of proteolytic enzymes that facilitates the penetration by the spermatozoa of the oocyte zona pellucida and reorganizes the sperm head in preparation for the sperm-oocyte fusion (30). Interestingly, although we found here an opposing role for ERK and p38 MAPK in motility, both are positively implicated in the acrosome reaction.

Sperm quality is determined by measuring the volume, sperm count, SM, FPM, morphology, and viability according to the World Health Organization guidelines (19). We therefore analyzed whether ERK1/2 and p38 MAPK (both the phosphorylated and the nonphosphorylated forms) can be correlated to the sperm quality parameters as above. Interestingly, total ERK1 and phospho-p38 levels were inversely correlated to the sperm quality parameters. Univariate analysis revealed that ERK1 and phospho-p38 could discriminate abnormal SM (<50%) and abnormal forward progression motility (FPM < 3) (19). Logistic regression (multivariate analysis) found ERK1 and phospho-p38 to be single and independent discriminators for

TABLE 3

|                | Area under the curve | Log cutoff | Sensitivity | Specificity | Area under the curve | Log cutoff | Sensitivity | Specificity |
|----------------|----------------------|------------|-------------|-------------|----------------------|------------|-------------|-------------|
| ERK1           | 0.727                | 1.56       | 45          | 100         | 0.821                | 0.55       | 68.4        | 92.9        |
| Phospho-p38    | 0.880                | 1.40       | 80          | 81.5        | 0.862                | 1.39       | 84.2        | 82.1        |

*This value is an average of an interval of values ranging from 0 to 1.1, in which the accuracy of measurement is constant.

FIGURE 10. ROC analysis for normal versus abnormal motility using phospho-p38 levels. Human semen samples were analyzed as in Table 1. Logarithmic transformation was used to normalize the distribution of enzyme levels. Using ROC analysis, a cut point of 1.40 for log-phospho-p38 could distinguish normal (>50%) from abnormal motility with 80% sensitivity and 81.5% specificity; AUC is 0.880.
ERK and p38 Are Regulators of Sperm Motility

![Diagram showing the role of ERK1/2 and p38 in human spermatozoan motility and acrosome reaction.](Image)

FIGURE 11. Proposed model for the role of ERK1/2 and p38 in human spermatozoan motility and acrosome reaction. ERK1/2 and p38 MAPK, but not JNK, are found in the tail of mature human spermatozoa. ERK1/2 stimulation is downstream to PKC (most likely PKCθ), which is also present in the human sperm tail (20). ERK stimulates and p38 inhibits forward and hyperactivated motility, respectively. Still, both ERK and p38 MAPK are positively involved in the acrosome reaction. ERK1/2 may exert its functions by phosphorylating ARHGAP6.

abnormal FPM and SM, respectively. ROC analysis revealed that phospho-p38 and ERK1 could diagnose abnormal FPM and reduced sperm motility with high specificity and sensitivity. Because ERK is positively involved in motility, and the majority of cellular ERK is in its nonphosphorylated form, a near constant phospho-ERK and a rise in its total form will reduce the specific activity and hence the inverse correlation. Because p38 is negatively involved in motility, it is therefore expected that p38 being nearly or fully phosphorylated (see “Results”) should display inverse correlation to motility as the data show. In most donors, we saw only ERK2, and the appearance of ERK1 predicted low sperm quality. MAPKs are thought to be regulated at the phosphorylation level and not via protein expression (7). Our results imply a possible pathological role for ERK1 gene expression. Mature spermatozoa are fully differentiated cells and do not have active transcriptional machinery, and ERK1 expression may have occurred during spermatogenesis. Alternatively, mature spermatozoa have an active mitochondrial translational apparatus (52); hence we do not rule out the possibility of ERK1 gene expression in mature spermatozoa with various degrees of asthenozoospermia. Indeed, ERK2 knock-out mice are embryonically lethal (53), whereas ERK1 knock-out mice are fertile (54). We assume that MEK1 is the major mediator of ERK1/2 activation in the sperm because MEK1 knock-out mice are embryonically lethal (55), whereas MEK2 knock-out mice are fertile (56).

The field of sperm biology and pathophysiology is certainly one of the least developed in reproduction research. However, male infertility because of sperm dysfunction (1 in 15 men) (57) and a general decrease in male fertility are growing areas in the field. To understand the underlying mechanisms involved in sperm dysfunction, one should first decipher the normal biochemical mechanisms of its functions. Our findings reported here shed further light on this basic aspect of human fertility. We have provided a potential molecular mechanism for the various forms of sperm motility (Fig. 11). Our data might be applied to unravel possible unbalanced expression of ERK1/2 and p38 in the various forms of male infertility (e.g. oligotestaenozoospermia). MAPK activators and inhibitors may be applied as a way to improve sperm quality of infertile men or when cryopreserved spermatozoa are used during assisted reproductive techniques. ERK1 and phospho-p38 MAPK can be contemplated as a means for spermatozoan diagnostics. Our studies might also pave the way for the potential use of MAPK activators, inhibitors, substrates, or sperm-specific MAPK-interacting proteins as potential tools for the development of nonhormonal contraceptives and immunon contraceptives for men and women.

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ERK and p38 Are Regulators of Sperm Motility

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