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# Phosphorylated Osteopontin Promotes Migration of Human Choriocarcinoma Cells via a p70 S6 Kinase-Dependent Pathway

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**Abstract** This study examined the role of osteopontin (OPN), a phosphorylated secreted glycoprotein, in the promotion of trophoblastic cell migration, an early event in the embryo implantation process. Three human choriocarcinoma cell lines, namely JAR, BeWo, and JEG-3, were treated with variants of OPN differing in the extent of phosphorylation following sequential dephosphorylation with tartrate-resistant acid phosphatase (TRAP), and their migratory response was measured. The highly phosphorylated human milk form of OPN (OPN-1) strongly triggered migration in all three cell lines, whereas the less phosphorylated variants, OPN-2a and OPN-2b, failed to stimulate migration. JAR cell migration in response to OPN-1 was accompanied by a rapid rearrangement of actin filaments to the cellular membrane. Using broad spectrum protein kinase profiling, we identified p70 S6 kinase as a major signal transduction pathway activated by OPN-1 during the migratory response in JAR cells. Activation was blocked completely by rapamycin and LY294002, thus demonstrating that OPN-1-stimulated migration occurs through mTOR and PI3K pathways, respectively. Conversely, PD98059 did not affect the activation of p70 S6 kinase by OPN-1, therefore, this response does not involve the Ras/ MAPK signaling cascade. Together, these data show that the highly phosphorylated human OPN-1 can stimulate trophoblastic cell migration and provides evidence for the involvement of the PI3K/mTOR/p70 S6 kinase pathway in the JAR cells response. Because both OPN and TRAP are expressed in the uterus during early pregnancy, it is conceivable that extracellular phosphatases such as TRAP may modify OPN charge state and thus modulate cell migration. J. Cell. Biochem. 94: 1218–1233, 2005. 

**Key words:** osteopontin; trophoblast; migration; p70 S6 kinase; uteroferrin; tartrate-resistant acid phosphatase

Several studies have shown that osteopontin (OPN), a non-collagenous phosphorylated glycoprotein originally isolated from bone extracellular matrix (ECM), plays an important role in embryo implantation by supporting attachment of trophoblastic cells to the epithelial lining of the endometrium [Garlow et al., 2002; Lessey, 2002; Apparao et al., 2003; Johnson et al., 2003; Weintraub et al., 2004], however, the role of OPN in facilitating the migratory function of these cells has not been previously studied. The phosphorylation state of OPN affects the migration or adhesion response of many cell types including osteoclasts [Ek-Rylander et al., 1994; Katayama et al., 1998; Razzouk et al., 2002], mammary epithelial cells [Tuck and Chambers, 2001], cancer cells [Furger et al., 2001; Standal et al., 2004], and cardiac smooth muscle cells [Jono et al., 2000]. In addition to its presence in bone, OPN is deposited as a prominent layer at the luminal surfaces of epithelial cells of the gastrointestinal

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tract, gall bladder, pancreas, urinary tract, placenta, lung, prostate, breast, glandular epithelia of the uterus, salivary glands, and sweat glands. It also has been localized in T-lymphocytes, macrophages, ganglion cells, and in vascular smooth muscle cells [Rodan, 1995]. In addition, OPN is found in biological fluids including milk [Bayless et al., 1997; Sorensen et al., 2003], urine [Yasui et al., 1999; Gang et al., 2001], and plasma [Koguchi et al., 2003; Standal et al., 2004], and it displays high levels of expression in sarcomas and carcinomas [Furger et al., 2001; Gaumann et al., 2001; Coppola et al., 2004].

The most frequently occurring post-translational modification in OPN is phosphorylation of the serine and threonine residues. Initial analyses of bone OPN showed that it contained an average of 12 phosphoserines (P-Ser) and 1 phosphothreonine (P-Thr) [Neame and Butler, 1996]. These represent an average value of the 25–27 individual sites that are partially phosphorylated in rat bone. Using highly phosphorylated bovine milk OPN, 27 P-Ser's, and 1 P-Thr residues were identified by amino acid sequencing and mass spectroscopic techniques. All, but two of the P-Ser's were present in the motif, Ser/Thr-X-Glu/P-Ser/Asp, and, thus, were in recognition sequences for mammary casein kinase [Sorensen et al., 1995]. The other two P-Ser's occurred within the sequence, Ser-X-X-Glu/P-Ser, both recognition sequences for casein kinase II [Wu et al., 1995; Lasa et al., 1997]. OPN also contains other consensus sequences for phosphorylation that can be recognized by other kinases. Salih et al. [1996] compared the phosphorylation potential of a series of kinases using in vitro $^{32}P$ labeling of bovine OPN and demonstrated that the factor-independent protein kinase (FIPK) incorporated 8.9 mol of $^{32}P$/mol into dephosphorylated OPN [Salih et al., 1996].

In addition, OPN has been identified as a potential substrate for the tartrate-resistant acid phosphatase (TRAP). Recently, it was suggested that TRAP could function as a regulator of OPN phosphorylation and bioactivity [Ek-Rylander et al., 1994; Andersson et al., 2003]. By comparing the results of Salih et al. [1996] with the identified TRAP-sensitive sites in OPN, it is intriguing to suggest that some of the phosphorylation of Ser 46 and 47, both potential TRAP dephosphorylation sites, occurs through FIPK [Salih et al., 1996].

Although OPN is encoded by a single gene, it occurs in multiple charge state forms that differ in the extent of phosphorylation [Safran et al., 1998]. Several lines of evidence demonstrate that the phosphorylation state modulates many functions including integrin-mediated adhesion [Ek-Rylander et al., 1994; Lecrone et al., 2000], mineralization [Zhu et al., 2001], bone resorption [Katayama et al., 1998; Razzouk et al., 2002], immune responses [Weber et al., 2002], and cellular migration [Andersson et al., 2003]. In osteoclasts, the binding of OPN to $\alpha_V\beta_3$ integrin promotes gelsolin associated signaling, leading to actin filament rearrangement [Chellaiah et al., 2000a, 2000b; Duong et al., 2000]. It has been suggested that the P-Ser's in OPN are required for the attachment of osteoclasts to plastic and bone slices and for the stimulation of bone resorption. Removal of phosphate from OPN by TRAP abolished its osteoclast attachment activity [Ek-Rylander et al., 1994]. Phosphorylation of recombinant OPN with CK II resulted in a two-fold increase of attachment of osteoclasts, whereas this difference had no effect on osteoblasts [Katayama et al., 1998]. In both of these studies, the exact sites of phosphorylation were not elucidated.

OPN stimulates migration of rat aortic smooth muscle cells in vitro [Yue et al., 1994], a response that depends, at least in part, on $\alpha_V\beta_3$ [Liaw et al., 1995]. These studies were performed with a mixture of OPN charge forms isolated from bone matrix. In addition, OPN can trigger chemotaxis or attachment through engagement of the homing receptor CD44 by a non-RGD cell-binding domain [van Dijk et al., 1993; Weber et al., 1996, 2002]. It thus appears that there may well be multiple interacting receptors for OPN, only some of which are RGD-dependent. Based on these collective findings, we predicted that OPN phosphorylation state would affect migratory responses in trophoblastic cells. This study was designed to understand the relationship between the phosphorylation state of OPN and the migration of human choriocarcinoma cells and to identify signaling pathways that mediate such responses.

## MATERIALS AND METHODS

### Materials

Rapamycin (FRAP/mTOR inhibitor), LY294002 (3-phosphoinositide-dependent protein kinase-

1 (PI3K) inhibitor), PD98059 (Raf/MEK inhibitor), and the PhosphoPlus® p70 S6 Kinase (Thr389, Thr421/Ser424) antibody kit were purchased from Cell Signaling Technology (Beverly, MA). p70 S6 kinase activity assay kit, p70 S6 kinase (T412E) active, and the rabbit anti-p70 S6 kinase antibody were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). [$\gamma$-$^{32}$P]ATP ~3,000 Ci/mmol was acquired from PerkinElmer (Wellesley, MA). The FITC-conjugated phalloidin was obtained from Sigma-Aldrich (St. Louis, MO). Recombinant human insulin-like growth factor II (IGF-II) was obtained from R&D Systems, Inc. (Minneapolis, MN). BCA Protein Assay kit was obtained from Pierce (Rockford, IL). CellTracker™ Green CMFDA was ordered from Molecular Probes (Eugene, OR). The cell sedimentation manifold was purchased from CSM, Inc. (Phoenix, AZ).

### OPN Charge Forms

The highly phosphorylated human milk OPN (called OPN-1 in this work) was purified essentially as described previously [Sorensen et al., 2003] except that an extra step of FPLC (Fast Performance Liquid Chromatography) gel filtration chromatography was included to achieve higher purity. Human milk OPN-1 was dephosphorylated by rat bone TRAP [1 mU/μg OPN] [Funhoff et al., 2001] at pH 5.0 for 15 or 120 min to achieve two lesser phosphorylated forms, termed OPN-2a and OPN2b, respectively. OPN-2a has 4–5 fewer P-Ser due to TRAP dephosphorylation, while OPN-2b is the product of further dephosphorylation. Table I and Figure 1 show that the dephosphorylated forms of OPN generated by a short (OPN-2a) and a long (OPN-2b) incubation with TRAP have lesser charge (higher isoelectric point [pI]) compared to the highly phosphorylated human milk OPN (OPN-1). These forms appear as a doublet before and after dephosphorylation with TRAP and thus are unlikely to represent phosphorylation variants. More likely they represent intact and an *N*-terminal fragment often seen in milk preparations. The extent of dye uptake is sensitive to phosphorylation state, a phenomenon that we observed previously with rat bone OPN [Safran et al., 1998].

### Isoelectric Focusing

The different phosphorylation forms of OPN (OPN-1, OPN-2a, and OPN-2b) were separated using the Novex® Pre-Cast IEF (Isoelectric Focusing) gels pH 3–7 from Invitrogen (Carlsbad, CA) following the manufacturer's instructions. In brief, 20 μg of protein was loaded in each lane of the gel and electrophoresed for 1 h at a constant 100 V, 1 h at a constant 200 V, and finally 30 min at 500 V. Upon reaching equilibrium, the gel was removed and washed three times with distilled water for 15 min, then fixed with 12% (w/v) trichloroacetic acid (TCA). The gel then was stained using GelCode® Blue staining reagent (Pierce). The migration of the charge forms of OPN then was compared to that of the isoelectric point standards, and the pIs were measured as described previously [Safran et al., 1998]. Note that the milk OPN charge forms are more acidic than the bone charge forms identified previously by this method (pI 4.6–5.1) [Safran et al., 1998].

### Cell Culture

The human trophoblastic cell lines, BeWo, JAR, and JEG-3, were purchased from ATCC (Manassas, VA). The BeWo, JAR, and JEG-3 cells were maintained, respectively, in Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate (ATCC), RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate (Invitrogen) and Eagle's Minimum essential medium with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate (ATCC) all supplemented with 10%

**TABLE I. Properties of OPN Phosphorylation Forms**

| OPN phosphorylation form | Description | Number of phosphates/molecule | pI |
|---|---|---|---|
| OPN-1 | High phosphate OPN, major form in human milk | 25 | 3.0 |
| OPN-2a | Medium phosphate OPN, obtained by in vitro dephosphorylation of OPN-1 by TRAP (short digestion) | 20–21 | 3.2 |
| OPN-2b | Low phosphate OPN, obtained by in vitro dephosphorylation of OPN-1 by TRAP (long digestion) | <20 | 3.35 |

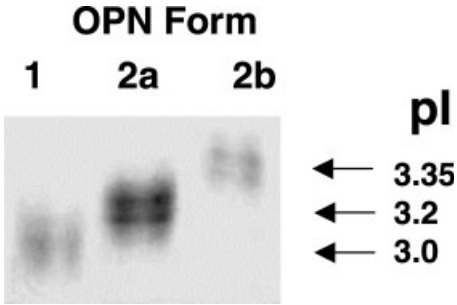


**Fig. 1.** Isoelectric focusing analysis of OPN phosphorylation variants. The sequential dephosphorylation of OPN-1(1) by TRAP produces two forms: OPN-2a (2a) and OPN-2b (2b). Although an equal amount of each phosphorylation form was used, the intensities of the bands vary because of variations in the ability of these forms to bind the GelCode® Blue staining reagent. The doublet is discussed in the text.

(v/v) charcoal-stripped fetal bovine serum (HyClone, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were seeded and maintained in Corning® 75 $cm^2$ rectangular culture flasks (Corning, Inc., Corning, NY) or six-well Costar® culture plates (Corning, Inc., Corning, NY) depending on the assay. Once the cell layer reached 80% confluency as judged by eye, the cells were grown in serum-free medium for 24 h before the experiments were initiated.

### Migration Assay

A "cell sedimentation manifold" and ten-well Teflon-coated microscopic slides were employed for this assay (CSM, Phoenix, AZ). Forty microliters of serum-free media were added to each well and the sedimentation manifold then was fixed to the slide. Cells were trypsinized in serum-free medium, counted, and fluorescently labeled using Cell Tracker™ Green CMFDA for 1 h following the manufacturer's instructions. At the end of the labeling period, the cells were gently rinsed several times with serum free media to remove excess dye. IGF-II was used as a positive control for human choriocarcinoma cell migration responses based on data from other studies [Chakraborty et al., 2002]. Cells then were treated with indicated concentrations of rapamycin for 30 min, LY294002 or PD98059 for 1 h as recommended by manufacturer. Forty microliters of serum-free media was added to each well and the sedimentation manifold was then fixed to the slide. One microliter of the cell suspension, containing 2,000 cells, then was injected into each well. The cells were allowed to attach for 2 h at 37°C in a humidified atmosphere of air/$CO_2$ (95:5 v/v). The manifold then was removed and the medium gently aspirated and replaced by the test treatment (OPN forms, IGF-II or serum-free media as the untreated control). Pictures of the cell aggregates were taken at this time point and then at various times during the course of the experiment using a Zeiss LSM 510 confocal microscope. Cell migration was measured and quantified using the LSM 510 software, which calculates the radius of migration based on the fluorescence intensity of cells. Any cell with intensity lower than ten (LSM 510 units) and that was not visible to the naked eye was not included in the radius. The average of the horizontal and vertical radii was calculated and the changes in the average radii at time point 24 h (T24) were determined. Statistical analyses were performed using one-way analysis of variance and the Tukey–Kramer multiple comparisons test (GraphPad InStat program, San Diego, CA).

### Immunofluorescence

JAR human choriocarcinoma cells were brought to 80% confluency in a Lab-Tek™ eight-chamber coverglass system (Nalge Nunc. International Corp.). The cells then were cultured in serum-free medium for 24 h, followed by a 24-h culture period either containing the indicated OPN form or no OPN as the untreated control. Cells were then fixed with 2% (v/v) formaldehyde (Sigma-Aldrich) in phosphate buffered saline (PBS) for 10 min followed by 0.1% (v/v) Triton X-100 (Sigma-Aldrich) in PBS. Cells then were washed three times, 5 min each, with PBS after which they were incubated for 1 h at 37°C with 5 μg/ml FITC-conjugated phalloidin (Sigma-Aldrich) then washed again with PBS. Nuclei then were stained by incubating cells with 0.5 μM Syto 59 (Molecular Probes) for 1 h at 37°C. This was followed by a final PBS wash. Pictures of the stained cells were taken using a Zeiss LSM 510 confocal microscope.

### KPKS Protein Kinase Profiling

JAR human choriocarcinoma cells were grown in serum-free medium for 24 h, then incubated with 75 ng/ml human milk OPN (OPN-1) for 90 min as recommended by Kinexus Bioinformatics Corp. (Vancouver, Canada). Proteins were then extracted following the Kinexus Bioinformatics Corp. protocol in ice-

cold lysis buffer containing protease inhibitors. The protein extracts from the OPN-treated and OPN-untreated samples were then processed following the company's recommendations and sent to Kinexus Bioinformatics Corp. for the Protein Kinase (KPKS 1.2) Screening. Results of this screening were provided in the form of Western blots and tables showing the quantitative analysis of these blots.

### Western Blotting

Protein samples from OPN-treated and untreated (used as a negative control) JAR cells incubated with or without the addition of rapamycin, LY294002 or PD98059 were prepared following the same procedure used for the protein kinase profiling. Protein concentrations were determined using the BCA protein assay (Pierce). Twenty micrograms of total protein was loaded in each well of the NuPAGE® Novex 10% (w/v) polyacrylamide bis–tris pre-cast gel (Invitrogen). Ten micrograms of total protein extracts from serum-treated NIH-3T3 cells was used as a positive control for the kit. The gel was then electrophoresed following the manufacturer's instructions. Proteins were then transferred to a Schleicher & Schuell Protran® nitrocellulose membrane (Intermountain Scientific; Kaysville, UT) at 4°C for 5 h at 40 V. The blots were processed based on the instructions from the PhosphoPlus® p70 S6 Kinase (Thr389, Thr421/Ser424) antibody kit (Cell Signaling Technology). Briefly, the blots were washed with Tris-Buffered Saline Tween-20 (TBST) for 5 min at room temperature (RT) followed by an incubation in blocking buffer (1× TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk) for 1 h at RT. Blots then were left in primary antibody overnight with gentle shaking at 4°C. Following a second wash, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody with gentle shaking for 1 h at RT. Finally, they were washed again and incubated with 10 ml LumiGlo® with gentle agitation for 1 min at RT. The blots then were exposed to X-ray film. Signal intensities were measured with the one-dimensional Multi Alpha Imager program (Alpha Innotech, San Leandro, CA) and the densitometric data were expressed as a percentage of the density of the p70 S6 kinase total protein band (detected using an antibody that recognizes total p70 S6 kinase protein) for the same sample. Statistical analyses were performed using one-way analysis of variance and the Tukey–Kramer multiple comparisons test (GraphPad InStat program).

### p70 S6 Kinase Activity Assay

p70 S6 kinase activity was measured using the p70 S6 kinase assay kit with an S6 peptide, AKRRRLSSLRA, used as a substrate in accordance with the procedure recommended by the manufacturer. Briefly, JAR cells were grown to 80% confluency as judged by eye in 100-mm dishes. The cells then were cultured without serum for 24 h followed by incubation with 75 ng/ml OPN-1, OPN-2a, OPN-2b or no additional treatment (untreated as a negative control) for 1 h. At the end of the treatment, the cells were lysed using 300 μl of the ice-cold lysis buffer described above. The cell lysates then were clarified by centrifugation at $17{,}000 \times g$ for 5 min at 4°C. The total protein concentrations of the supernatant fractions were determined using the BCA protein assay (Pierce). Four micrograms of anti-p70 S6 kinase antibody were mixed with 100 μl of a 50% protein A agarose slurry that had been washed in the lysis buffer. Cell lysate (1 mg) or 100 mU of the active p70 S6 kinase then was added to the beads followed by incubation for 2 h at 4°C to immunopurify the enzyme. The protein A-agarose/enzyme immunocomplex then was washed in 500 μl lysis buffer three times and then once in 500 μl Assay Dilution Buffer I (ADBI). Thirty microliters of ADBI was added to the microfuge tube containing the protein A enzyme immunocomplex followed by 10 μl of inhibitor cocktail, 10 μl substrate cocktail, and 10 μl [$\gamma$-$^{32}$P]-conjugated adenosine triphosphate ([$\gamma$-$^{32}$P]-ATP) mixture. At the end of a 10 min incubation at 30°C, the reaction was stopped by transferring a 25 μl aliquot onto the center of a 2 × 2 cm P81 paper. The assay squares then were washed three times with 40 ml 0.75% (v/v) phosphoric acid for 5 min per wash and once with acetone for 5 min. The assay squares then were transferred to a scintillation vial and 5 ml scintillation cocktail was added and then counted in a scintillation counter. At the same time, immunoprecipitates isolated by non-immune rabbit IgG serum (Sigma-Aldrich) instead of the p70 S6 kinase antibody were used as negative controls. The radioactivity (in dpm) of the active enzyme samples was compared to radioactivity of control samples that did not contain enzyme (background control). After subtraction of background and IgG control dpm from each of the

samples, the OPN-1 stimulated p70 S6 kinase activity was normalized to untreated controls and expressed as a fold change. The experiments were repeated and statistical analyses were performed using one-way analysis of variance and the Tukey–Kramer multiple comparisons test (GraphPad InStat program).

## RESULTS

### Osteopontin Induces JAR Human Choriocarcinoma Cells Migration in a Phosphorylation-Dependent Manner

Our first objective was to determine if TRAP dephosphorylation of OPN affected migration responses in human choriocarcinoma cells. We compared the relative abilities of each of the OPN phosphorylation variants to stimulate migration using three different human choriocarcinoma cell lines: JAR, BeWo, and JEG-3. Following a 24-h serum withdrawal, the cells were plated for the migration assay as described in the experimental procedures. JAR cells initially were used to determine the appropriate OPN concentration. Preliminary OPN-1 concentration-dependence and time course experiments showed that the optimal conditions for stimulating cellular migration were 75 ng/ml (approximately 2 nM) and treatment for 24 h (data not shown). As shown in Figure 2A, OPN-1 strongly induced migration of JAR cells, measured as an increase in circle diameter, over a 24 h treatment period, whereas the dephosphorylated OPN-2a and OPN-2b forms triggered only a mild response as compared to the untreated cells (Fig. 2B). The data shown in Figure 2C,D demonstrate that similar responses occurred in two other choriocarcinoma cell lines, namely BeWo and JEG-3. These data indicate that fully phosphorylated OPN-1 is an effective stimulator of cell migration, while TRAP dephosphorylation markedly diminishes this activity in all three human choriocarcinoma cell lines that we tested (data not shown).

In order to determine if the increase in the diameter of the circles used to follow cell migration might be due to proliferation, a proliferation assay in which we directly counted number

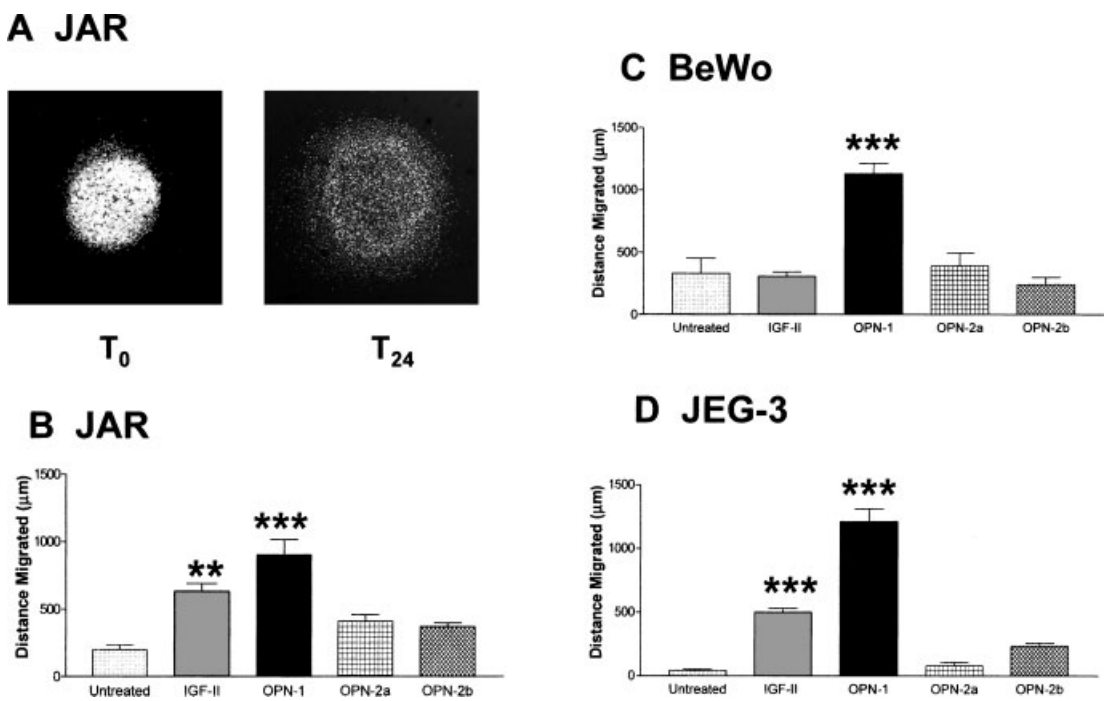


**Fig. 2.** The highly phosphorylated OPN-1 stimulates migration of human choriocarcinoma cell lines. **A**, **B**: JAR cell migration measured after 24 h treatment with 75 ng/ml OPN-1. Migration was measured by subtracting the average radius (between horizontal and vertical radius) at time zero ($T_0$) from the average radius at the 24 h time point ($T_{24}$). The same procedure was used to measure migration of BeWo (**C**) and JEG-3 (**D**) cells in response to 24 h treatment with 75 ng/ml OPN-1. IGF-II was used as a positive control for stimulating migration of the three cell lines mentioned above, although the BeWo cells failed to respond to it. The results represent the average ± SD of three independent experiments. **, $P < 0.01$; ***, $P < 0.001$ compared to untreated sample.

of JAR cells was performed. The cell number was not significantly different after treatment with OPN-1, indicating that migration most likely accounted for the increase in diameter of the circle (data not shown). Our next step was to determine if the migratory response triggered by OPN-1 was correlated to a cytoskeletal rearrangement. As shown in Figure 3, after treatment with OPN-1, there was a clearly visible recruitment of actin filaments to the periphery of the JAR cells, subjacent to the plasma membrane. OPN-2a and OPN-2b treatment did not produce these dramatic changes, although some increase in staining at the periphery was evident. Interestingly, even though the total number of JAR cells was not statistically different, some cell division was evident (see panel with OPN-1, arrow).

### OPN-1, but not OPN-2a and OPN-2b, Induces Phosphorylation and Activation of p70 S6 Kinase

Activation of cell migration is expected to be accompanied by activation of intracellular signaling cascades. Therefore, we examined, which signal transduction pathways might be activated as a consequence of OPN-1 treatment using a broad screening approach. JAR cells were treated with 75 ng/ml OPN-1 for 90 min at 37°C, conditions recommended to us by the Kinexus group to permit broad kinase screening, to identify the signaling pathways that induce migration. The cell extracts then were analyzed off site using their protein kinase array. Table II shows the results of this analysis. Almost all of the 55 kinases that were assayed changed modestly or even decreased in

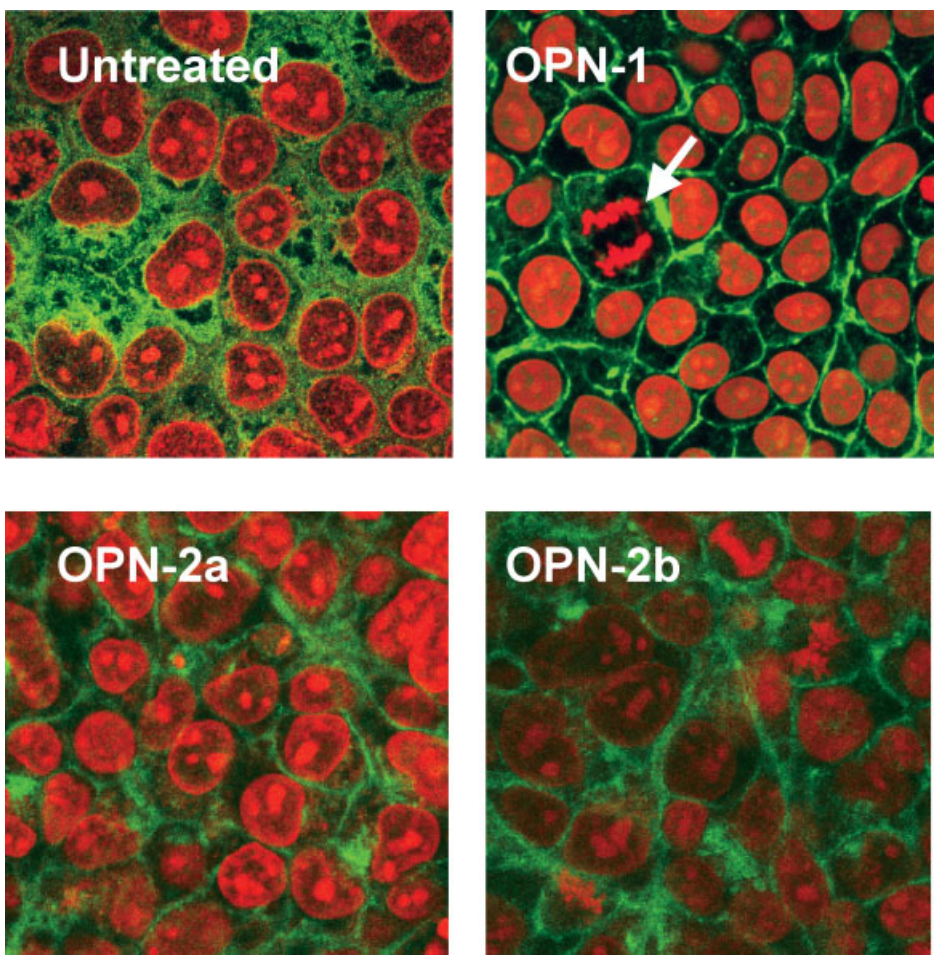


**Fig. 3.** OPN-1 triggers actin filament rearrangement in JAR human choriocarcinoma cells. Using FITC-conjugated phalloidin, actin filaments from cells treated with 75 ng/ml of the different forms of OPN for 24 h were visualized. Note the recruitment of the actin filaments to the cell periphery upon treatment with OPN-1, but to a much lesser extent with OPN-2a and OPN-2b.

**TABLE II. KPKS Protein Kinase Profiling of OPN-1 Treated JAR Human Choriocarcinoma Cells***

| Full name of protein | Untreated | OPN-1 | Ratio OPN-1/ untreated |
|---|---|---|---|
| Aurora2 | 427 | 800 | 1.9 |
| Cancer Osaka thyroid oncogene (Tpl2) | 1,032 | 970 | 0.9 |
| Casein kinase 2 (35) | 8,068 | 5,781 | 0.7 |
| Casein kinase 2 (37) | 3,319 | 4,180 | 1.3 |
| Casein kinase 2 (39) | 7,866 | 6,201 | 0.8 |
| Cyclin-dependent kinase 1 (cdc2) | 452 | 398 | 0.9 |
| Cyclin-dependent kinase 2 | 1,568 | 1,128 | 0.7 |
| Cyclin-dependent kinase 4 | 311 | 297 | 1.0 |
| Cyclin-dependent kinase 7 | 1,813 | 1,787 | 1.0 |
| Cyclin-dependent kinase 9 | 2,951 | 3,737 | 1.3 |
| DNA-activated protein kinase | 605 | 901 | 1.5 |
| Extracellular regulated kinase 1 | 12,380 | 7,645 | 0.6 |
| Extracellular regulated kinase 1 (41) | 514 | 492 | 1.0 |
| Extracellular regulated kinase 2 (37) | 1,630 | 1,189 | 0.7 |
| Extracellular regulated kinase 2 (37) | 2,837 | 1,832 | 0.6 |
| Extracellular regulated kinase 2 | 346 | 436 | 1.3 |
| Extracellular regulated kinase 3 | 1,379 | 620 | 0.4 |
| Glycogen synthase kinase 3 alpha (44) | 558 | 482 | 0.9 |
| Glycogen synthase kinase 3 beta (40) | 507 | 551 | 1.1 |
| MAP kinase kinase 1 (MKK1) | 1,729 | 1,699 | 1.0 |
| MAP kinase kinase 2 (MKK2) | 1,503 | 1,661 | 1.1 |
| MAP Kinase Kinase 6 (MEK6) | 6,777 | 6,921 | 1.0 |
| p21 activated Kinase 3 (PAK beta) | 8,463 | 7,886 | 0.9 |
| p38 Hog MAP kinase | 6,105 | 3,616 | 0.6 |
| Protein kinase B alpha | 466 | 867 | 1.9 |
| Protein kinase C alpha | 340 | 259 | 0.8 |
| Protein kinase C beta1 | 3,536 | 2,592 | 0.7 |
| Protein kinase C mu (110) | 269 | 284 | 1.1 |
| Ribosomal S6 kinase 1 (78) | 1,267 | 2,838 | 2.2 |
| Ribosomal S6 kinase 2 (73) | 850 | 1,383 | 1.6 |
| S6 Kinase p70 | 378 | 1,109 | 2.9 |
| Stress activated protein kinase (JNK) (45) | 593 | 889 | 1.5 |
| v-mos Moloney murine sarcoma viral oncogene homolog 1 (34) | 312 | 578 | 1.9 |
| v-raf murine sarcoma viral oncogene homolog B1 (92) | 4,150 | 4,297 | 1.0 |
| Calmodulin-dependent kinase 1 | 277 | 279 | 1.0 |
| Casein kinase 1 delta | 169 | 99 | 0.6 |
| Casein kinase 1 epsilon | 236 | 159 | 0.7 |
| c-SRC tyrosine kinase | 257 | 166 | 0.6 |
| Death associated protein kinase 1 | 1,075 | 1,355 | 1.3 |
| Focal adhesion kinase | 767 | 600 | 0.8 |
| G protein-coupled receptor kinase 2 (BARK2) | 723 | 534 | 0.7 |
| Germinal center kinase | 189 | 184 | 1.0 |
| Inhibitor NF kB kinase alpha/beta | 1,136 | 1,430 | 1.3 |
| Janus kinase 1 | 1,077 | 518 | 0.5 |
| Janus kinase 2 | 57 | 62 | 1.1 |
| Mammalian sterile 20-like 1 | 183 | 124 | 0.7 |
| Oncogene Lyn | 294 | 361 | 1.2 |
| Oncogene Raf 1 (69) | 450 | 552 | 1.2 |
| Oncogene Raf 1 (73) | 2,860 | 3,406 | 1.2 |
| Oncogene SRC | 408 | 172 | 0.4 |
| Protein kinase G1 (cGMP-dependent protein kinase) | 72 | 53 | 0.7 |
| Yamaguchi sarcoma viral oncogene homolog 1 | 180 | 60 | 0.3 |
| Zeta-chain (TCR) associated protein kinase | 378 | 275 | 0.7 |
| ZIP kinase (death associated protein kinase 3) | 2,656 | 3,075 | 1.2 |

*The trace quantity units are arbitrary based on the intensity of ECL fluorescence detection for target immunoreactive proteins recorded with a Bio-Rad Fluor-S MultiImager and Quantity One software. Values are the average of duplicate determinations. In the case of multiple variants for a kinase, the molecular weights of the multiple bands are presented as numbers in kilodaltons after the full name of the protein.
KPKS, kinetworks protein kinase screen.

response to OPN-1; however, p70 S6 kinase increased almost three-fold in response to OPN-1. Based on this result, we performed additional experiments to validate this finding. Because we thought it likely that the 90 min period of the screening assay might be at the tail end of a rapidly activated signaling pathway, a time course was performed to determine the kinetics of OPN-1 stimulated phosphorylation of p70 S6 kinase. Figure 4 shows that the maximal phosphorylation of p70 S6 kinase in response to OPN-1 occurred after 1 h of treatment for both of the target phosphorylation sites, Thr-389 (A) and Thr-421/Ser-424 (B) while the total levels of endogenous p70 S6 kinase remained stable throughout the experiment (Fig. 4C). This latter

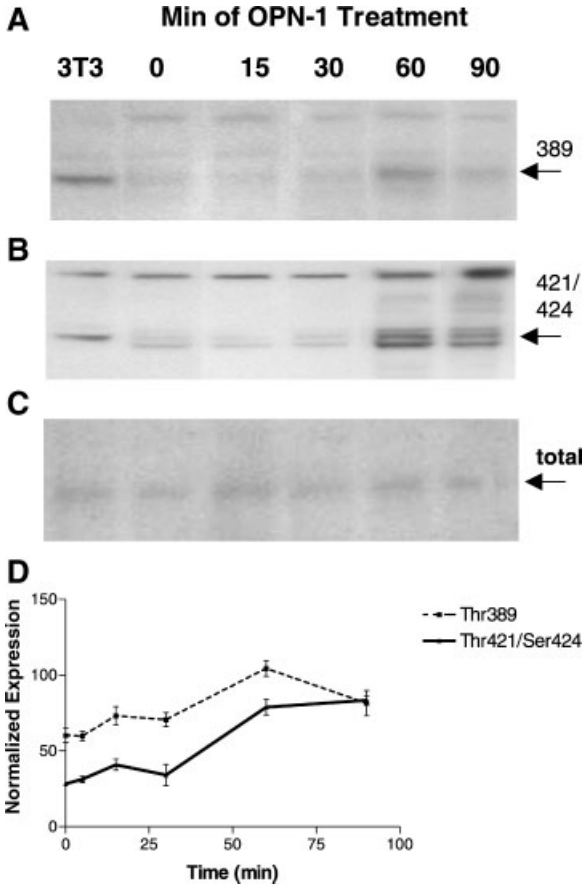


**Fig. 4.** Time-dependent stimulation of p70 S6 kinase phosphorylation at Thr-389, and Thr-421/Ser-424 sites by OPN-1. Western blot analysis of cell extracts from OPN-1 treated (75 ng/ml) JAR human choriocarcinoma cells. The blots were probed with antibodies that specifically recognize phosphorylated at Thr-389 (**A, D**) and Thr-421/Ser-424 (**B, D**) and total p70 S6 kinase protein (**C**). NIH-3T3 cell extracts treated with serum were used as the positive control and were provided with the PhosphoPlus® p70 S6 kinase antibody kit. The experiments were performed in triplicate and repeated three times with similar results. Quantification of the data panels A–C is shown in panel D.

result indicated that the observed increases in phosphorylation are not due solely to increases in protein expression. Consistent with the failure to induce migration, OPN-2a and OPN-2b did not activate p70 S6 kinase at either site of phosphorylation and neither affected the total protein levels of p70 S6 kinase (data not shown).

Because phosphorylation of these two critical regions is a requirement for the activation of p70 S6 kinase, it was reasonable to expect that inhibition of these phosphorylation events might interfere with OPN-1 induced migration. For all subsequent studies with various kinase inhibitors, we chose 1 h of OPN-1 treatment, because this time correlated with maximal phosphorylation at both sites. In order to further determine if specific phosphorylation events were leading to the activation of p70 S6 kinase when JAR human choriocarcinoma cells were treated for 1 h with OPN-1, a direct kinase activity assay was performed using a peptide substrate specifically phosphorylated by p70 S6 kinase, AKRRRLSSLRA (see Methods). The results of this assay are shown in Figure 5 and clearly demonstrated that p70 S6 kinase activity is activated at least two-fold when choriocarcinoma cells are treated with OPN-1, and to a much lesser extent when the other OPN forms were used.

### Rapamycin and LY294002, but not PD98059, Block the Migratory Effect of OPN-1 in JAR Human Choriocarcinoma Cells

To complement the studies on p70 S6 kinase activation, we investigated whether kinase inhibitors would inhibit OPN-1 stimulated migration. Since, p70 S6 kinase can be phosphorylated either through the PI3K/mTOR pathway or the Raf/MEK pathway, we used specific inhibitors for each pathway to determine their relative contributions to the response. The optimal concentrations for these inhibitors were identified in preliminary dose response experiments (data not shown). Rapamycin, a potent inhibitor of the activation of p70

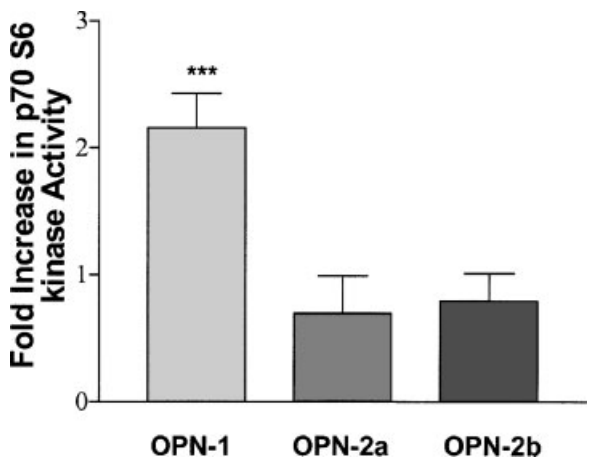


**Fig. 5.** OPN increases p70 S6 kinase activity in a phosphorylation dependent manner. Using a p70 S6 kinase activity assay, OPN-1 treatment for 1 h was found to increase the activity of this kinase approximately two-fold compared to untreated cells while OPN-2a and OPN-2b had little effect. The active S6 kinase was used as a positive control (data not shown). The experiments were performed in triplicate and repeated three times with similar results. ***, $P < 0.001$ compared to untreated sample.

S6 kinase, completely blocked OPN-1 stimulated JAR cell migration (Fig. 6A). The inhibition of OPN-1 induced migration by rapamycin was statistically significant, consistent with mTOR (mammalian Target of Rapamycin) as an upstream activator of p70 S6 kinase. Likewise, LY294002, a selective PI3 kinase (PI3K) inhibitor, blocked the OPN-1 stimulated migratory response (Fig. 6B), thus demonstrating the involvement of PI3K in the pathway leading to JAR cell migration and p70 S6 kinase activation. In contrast, PD98059, a MEK1 inhibitor of MAP kinase (MAPK) activation, had no inhibitory effect on migration (Fig. 6C). Collectively, these results indicate that both PI3K and mTOR pathways, acting through p70 S6 kinase, were likely to be activated by OPN-1 and involved in the stimulation of JAR cell migration.

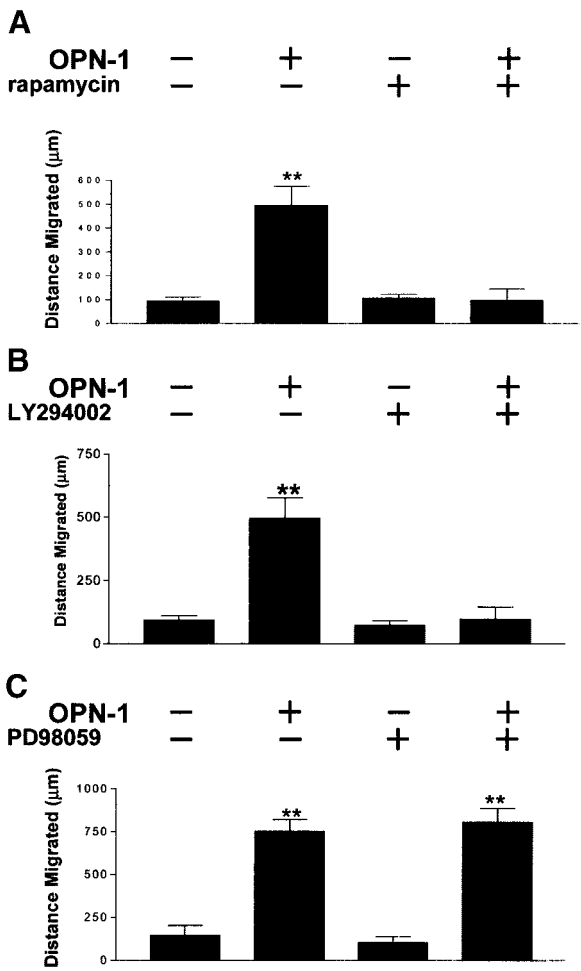


**Fig. 6.** OPN-1 stimulation of JAR cell migration is inhibited by rapamycin and LY294002, but not PD98059. OPN-1 was added at 75 ng/ml in all experiments, which were conducted over a 24 h migration period. **A**: JAR cells were pre-treated with 10 nM rapamycin for 30 min before the start of the migration assay. **B**: JAR cells were pre-treated with 20 μm LY294002 for 1 h before the start of the migration assay. **C**: JAR cells were pre-treated with 50 μm PD98059 for 1 h before the start of the migration assay. The results represent the average ± SD of three independent experiments. **, $P < 0.01$.

### OPN-1 Induces Phosphorylation of p70 S6 Kinase Through the PI3K/mTOR

Having determined the effect of specific inhibitors on migratory responses, our next goal was to definitively link the p70 S6 pathway to the migratory response. To accomplish this, we used the same inhibitors used to inhibit migration, but this time tested their effects on phosphorylation of p70 S6 kinase. As shown in Figure 7, the addition of rapamycin (10 nM) completely inhibited the phosphorylation of p70 S6 kinase at Thr-389 (Fig. 7A) and Thr 421/Ser 424 (Fig. 7B) to levels that were not significantly different from untreated cells. This abolishment of the OPN-1 induced activation of p70 S6 kinase by rapamycin indicated that the activation of this kinase by OPN-1 is achieved through the activation of mTOR. No effect on total p70 S6 kinase levels was observed (Fig. 7C). In a similar fashion, LY294002 significantly blocked phosphorylation at Thr-389 (Fig. 8A) and the Thr-421/Ser-424 sites (Fig. 8B), thus showing that PI3K acts as an upstream activator of mTOR and subsequently p70 S6 kinase in choriocarcinoma cells treated with OPN-1. As with rapamycin, there was no effect on total protein levels (Fig. 8C). In contrast, treatment with 50 μM of the MAPK pathway inhibitor, PD98059, for 1 h did not affect the phosphorylation of p70 S6 kinase at either site (Fig. 9A,B). No effect was seen on levels of p70 S6 kinase (Fig. 9C). In additional experiments, we further showed that TRAP dephosphorylated OPN forms, OPN-2a and OPN-2b, did not stimulate p70 S6 kinase phosphorylation at any site (data not shown). Together these data suggest that OPN-1, but not OPN-2a or 2b, induces phosphorylation of mTOR through the PI3 kinase pathway and that OPN-1 activates p70 S6 kinase via the PI3K/mTOR pathway, but not the Raf/MEK pathway, in the JAR human choriocarcinoma cells.

## DISCUSSION

OPN expression has been correlated with the onset of cell attachment competence during the

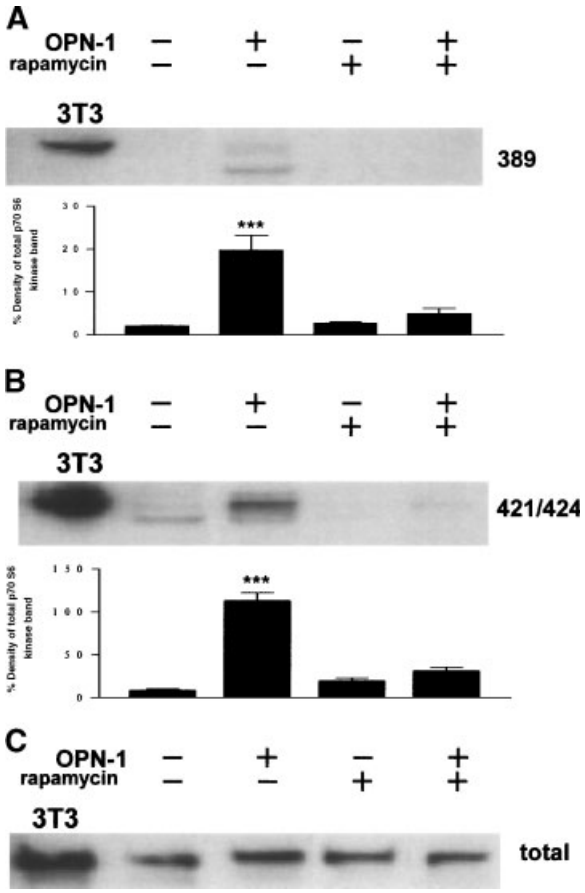


**Fig. 7.** mTOR inhibition by rapamycin inhibits phosphorylation of p70 S6 kinase. In all panels, cell lysates were prepared from JAR cells subjected to the indicated treatments. Rapamycin (10 nM) or vehicle was added for a 30 min pre-treatment, followed by a 1 h treatment at 37°C with 75 ng/ml OPN-1 or left untreated. Western blots were probed with antibodies that specifically recognize phosphorylated p70 S6 kinase phosphorylated at Thr-389 (**A**) Thr-421/Ser-424 (**B**) or total p70 S6 kinase protein present in cell extracts (**C**) as described in Materials and Methods. NIH-3T3 cells treated with serum were used as the positive control and were provided with the PhosphoPlus® p70 S6 kinase antibody kit. All experiments were performed in triplicate and repeated three times with similar results. ***, $P < 0.001$ for OPN-1 treated compared to untreated sample. There was no significant difference between untreated and rapamycin treated samples.

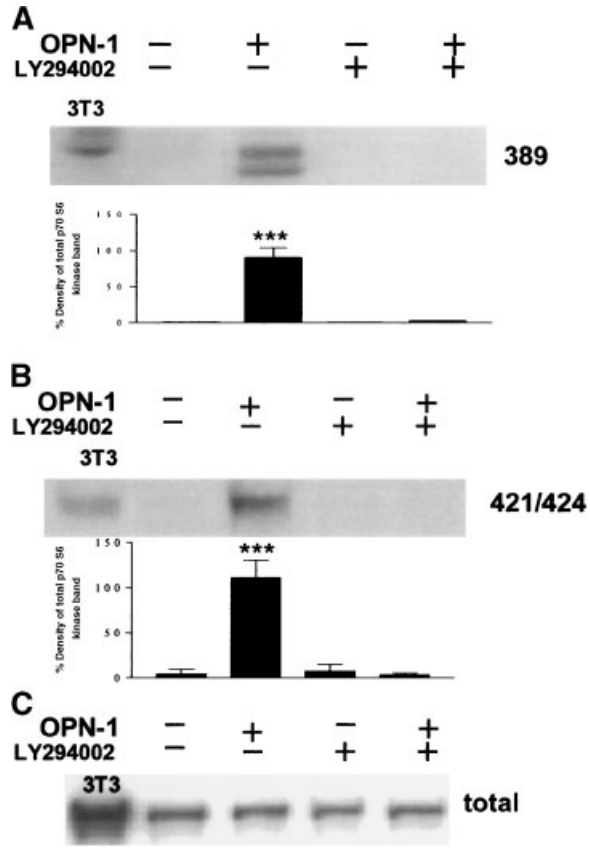


**Fig. 8.** PI3K inhibition by LY294002 inhibits p70 S6 kinase phosphorylation. In all panels, cell lysates were prepared from JAR cells subjected to the indicated treatments. LY294002 (20 μm) or vehicle was added for a 1 h pre-treatment, followed by a 1 h treatment at 37°C with 75 ng/ml OPN-1 or left untreated. Western blots were probed with antibodies that specifically recognize phosphorylated p70 S6 kinase phosphorylated at Thr-389 (**A**) Thr-421/Ser-424 (**B**) or total p70 S6 kinase protein present in cell extracts (**C**) as described in Materials and Methods. NIH-3T3 cells treated with serum were used as the positive control and were provided with the PhosphoPlus® p70 S6 kinase antibody kit. All experiments were performed in triplicate and repeated three times with similar results. ***, $P < 0.001$ for OPN-1 treated compared to untreated sample. There was no significant difference between untreated and LY294002 treated samples.

implantation process, taking place at the endometrial/ trophoblastic interface in several species including humans [Lessey, 2002]. In this regard, OPN has been considered to function primarily as an attachment facilitator for trophoblasts rather than as a trigger for migration and invasion into the uterine stroma. The purpose of this study was to determine if OPN could trigger cell migration in human trophoblastic cell lines, and if OPN charge forms differing in the extent of phosphorylation had different activities. Our data demonstrate clearly that fully phosphorylated OPN-1, in contrast with TRAP dephosphorylated forms, potently stimulates migration of three trophoblastic lines that we tested. Furthermore, we showed that OPN-1, but not OPN-2a and OPN-2b, triggers cell migration by activating a p70 S6 kinase dependent pathway through PI3 kinase and mTOR. These are significant findings because they demonstrate the importance of OPN phosphorylation in the regulation of OPN bioactivity. Furthermore, the results indicate that dynamic dephosphorylation of OPN-1 by

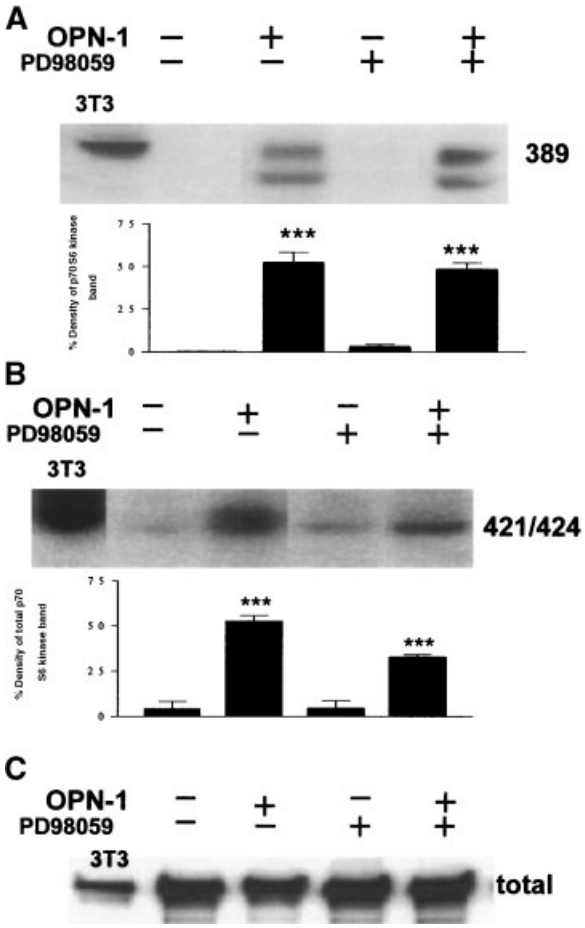


**Fig. 9.** MEK/MAPK pathway inhibition by PD98059 does not inhibit p70 S6 kinase activation in response to OPN-1. In all panels, cell lysates were prepared from JAR cells subjected to the indicated treatments. PD98059 (50 μm) or vehicle was added for a 1 h pre-treatment, followed by a 1 h treatment at 37°C with 75 ng/ml OPN-1 or left untreated. Western blots were probed with antibodies that specifically recognize phosphorylated p70 S6 kinase phosphorylated at Thr-389 (**A**) Thr-421/Ser-424 (**B**) or total p70 S6 kinase protein present in cell extracts (**C**) as described in Materials and Methods. NIH-3T3 cells treated with serum were used as the positive control and were provided with the PhosphoPlus® p70 S6 kinase antibody kit. All experiments were performed in triplicate and repeated three times with similar results. ***, $P < 0.001$ for OPN-1 treated compared to untreated sample or OPN-1 plus PD98059 compared to PD98059 only.

TRAP can attenuate cell migration capacity in this cell type, a finding of interest given the co-expression of these two molecules in the uterus during early pregnancy [Lessey, 2002; Johnson et al., 2003].

In the current study, we used OPN phosphorylation forms that are well-characterized biochemically (analysis performed by Dr. Esben S. Sorensen, data not shown). The OPN phosphorylation forms from human milk have lower pIs than those reported previously for OPN forms from bone [Safran et al., 1998]. Our data suggest a requirement for high level phosphorylation of OPN, such as in OPN-1 from milk, for the induction of JAR human choriocarcinoma cells migration. This effect is not cell line specific, because it also was observed in the other two human choriocarcinoma cell lines, BeWo and JEG-3. Partial dephosphorylation of OPN-1 by TRAP produced the novel forms OPN-2a and OPN-2b and abolished the migratory response. This suggested that the loss of specific phosphates might affect the ability of the protein to interact with cell surface receptor binding site(s) and trigger signals involved in migration. We are presently exploring this idea further, focusing first on the known receptors for OPN, namely $\alpha_v\beta_3$ integrin or CD44 [Xie et al., 2001; Lessey, 2002]. There is evidence of the involvement of $\beta_3$ integrin in activating this pathway in adult cardiac muscle and endothelial cells [Balasubramanian and Kuppuswamy, 2003; Sudhakar et al., 2003], however CD44 has not been implicated in this process. Either of these could be the receptor responsible for mediating the OPN-1 activation of migration in human choriocarcinoma cells; however a synergy between the two receptors is also possible.

Recent studies suggest that p70 S6 kinase phosphorylation induces migration of vascular smooth muscle cells, neutrophils, and chicken embryo fibroblasts through the PI3K/mTOR pathway [Goncharova et al., 2002; Gomez-Cambronero et al., 2003; Qian et al., 2004]; however, there have been no reports linking OPN and p70 S6 kinase phosphorylation during a migratory response. p70 S6 kinase is a Ser/Thr protein kinase whose activation is involved in diverse cellular responses, such as the $G_1$ to S phase transition in cell proliferation, migration, and responses to different growth factors and hormones [Pullen and Thomas, 1997; Goncharova et al., 2002; Huang and Kontos, 2002; Gomez-Cambronero, 2003; Gomez-Cambronero et al., 2003]. Activation of p70 S6 kinase is achieved through a signaling pathway downstream of Akt and PI3K. This activation leads to the phosphorylation of the ribosomal protein S6 which, in turn, enhances translation of 5′-pyrimidine tract mRNAs. The ribosomal protein S6 has been shown to be phosphorylated by two major kinases, namely the p90 rsk and p70 S6 kinase. While the first one is active for only a few minutes, p70 S6

kinase takes around an hour to be activated and is essentially responsible for the sustained phase activity of ribosomal protein S6 [Sweet et al., 1990; Chen et al., 1991; Kahan et al., 1992]. This event is controlled by a complex process involving the hierarchical phosphorylation of multiple sites in p70 S6 kinase and the activation of rapamycin-sensitive mTOR [Pullen and Thomas, 1997]. The activation of the autoinhibitory domain (AID), where the Thr421/Ser424 site is located, is believed to precede that of the other regulatory sites in this kinase. This activation involves the relief of pseudosubstrate suppression, an effect that is blocked by rapamycin [Alessi et al., 1998; Polakiewicz et al., 1998; Weng et al., 1998]. The phosphorylation sites in the AID are found in a consensus motif similar to that recognized by the mitogen-activated protein kinases (MAPK/Erk). Although MAPK can phosphorylate p70 S6 kinase, it has been demonstrated that this event is neither necessary nor sufficient for the activation of the latter [Pullen and Thomas, 1997]. The phosphorylation of the Thr389 site in the linker domain, which connects the AID to the catalytic domain of the kinase, appears to be the next event associated with the activation of p70 S6 kinase. Dephosphorylation of this site is thought to be closely related to the loss of kinase activity. Phosphorylation of the Thr389 in a PI3K-dependent manner leads to phosphorylation of the Thr229 site in the catalytic domain by PDK-1 through PKCζ, thus, activating the kinase [Dennis et al., 1996, 1998; Pullen and Thomas, 1997; Alessi et al., 1998; Pullen et al., 1998; Saitoh et al., 2002]. This activation event can be inhibited by the PI3K inhibitor, LY294002, or the mTOR inhibitor, rapamycin [Pullen and Thomas, 1997; Gomez-Cambronero, 2003]. In this study, we investigated the role of this pathway in the migratory responses of JAR cells to various charge forms of OPN differing in the extent of phosphorylation.

The results of this study demonstrate for the first time that p70 S6 kinase is activated by OPN-1 and is involved in human choriocarcinoma cell migration. Our results indicate that treatment of JAR cells with OPN-1 leads to the phosphorylation of both the Thr389 and Thr421/Ser424 sites on p70 S6 kinase within 1 h. The TRAP dephosphorylated OPN forms, OPN-2a and OPN-2b, did not stimulate p70 S6 kinase phosphorylation, thus suggesting that there is a requirement for a high level of phosphorylation of OPN for the activation of this signaling pathway. When cells were pretreated with rapamycin, they did not migrate in response to OPN-1. Phosphorylation of p70 S6 kinase at the Thr389 and the Thr421/Ser424 sites was also completely blocked by rapamycin. These results suggest that the p70 S6 kinase activation after OPN-1 treatment involves the activation of the rapamycin-sensitive mTOR kinase. Activation of p70 S6 kinase in response to OPN-1 also is inhibited by the PI3K inhibitor, LY294002, and is not affected by the Raf/MEK inhibitor, PD98059. JAR migration in response to OPN-1 also was blocked completely by LY294002. Therefore, p70 S6 kinase activation and JAR cell migration induced by OPN-1 is mediated by PI3K rather than by the Ras/MAPK cascade. This finding is consistent with other studies on the activation of p70 S6 kinase during migratory responses [Gomez-Cambronero, 2003; Gomez-Cambronero et al., 2003; Berven et al., 2004].

Of further interest, the type 5, iron-containing, TRAP constitutes a relatively minor intracellular isozyme of acid phosphatase in the human that is identical to uteroferrin, a secreted progesterone-induced protein of the porcine uterus [Gonzalez et al., 1995]. TRAP has been shown to be expressed in the human placenta [Janckila et al., 1996]. The activity of acid phosphatase in the human endometrium is slightly lower during the proliferative phase than is that of alkaline phosphatase. However the activity of acid phosphatase has been shown to increase rapidly during the window of implantation and to remain high through early pregnancy, while the activity of alkaline phosphatase remains constant [Ganguly and Ghosh, 1978]. Our findings suggest that TRAP activity, through dephosphorylation of OPN, might affect the ability of OPN to trigger trophoblast cell migration. While the form of OPN secreted by the human uterus has never been demonstrated, many secretory epithelia including breast and kidney secrete highly phosphorylated forms of OPN [Rodan, 1995]. On this basis, it is intriguing to speculate that TRAP could regulate the ability of OPN to trigger trophoblast cell migration during the implantation process by changing OPN phosphorylation level. Based on the data presented in this study, we propose a model of action for OPN-1 on JAR cells as presented in Figure 10. Our results reveal a

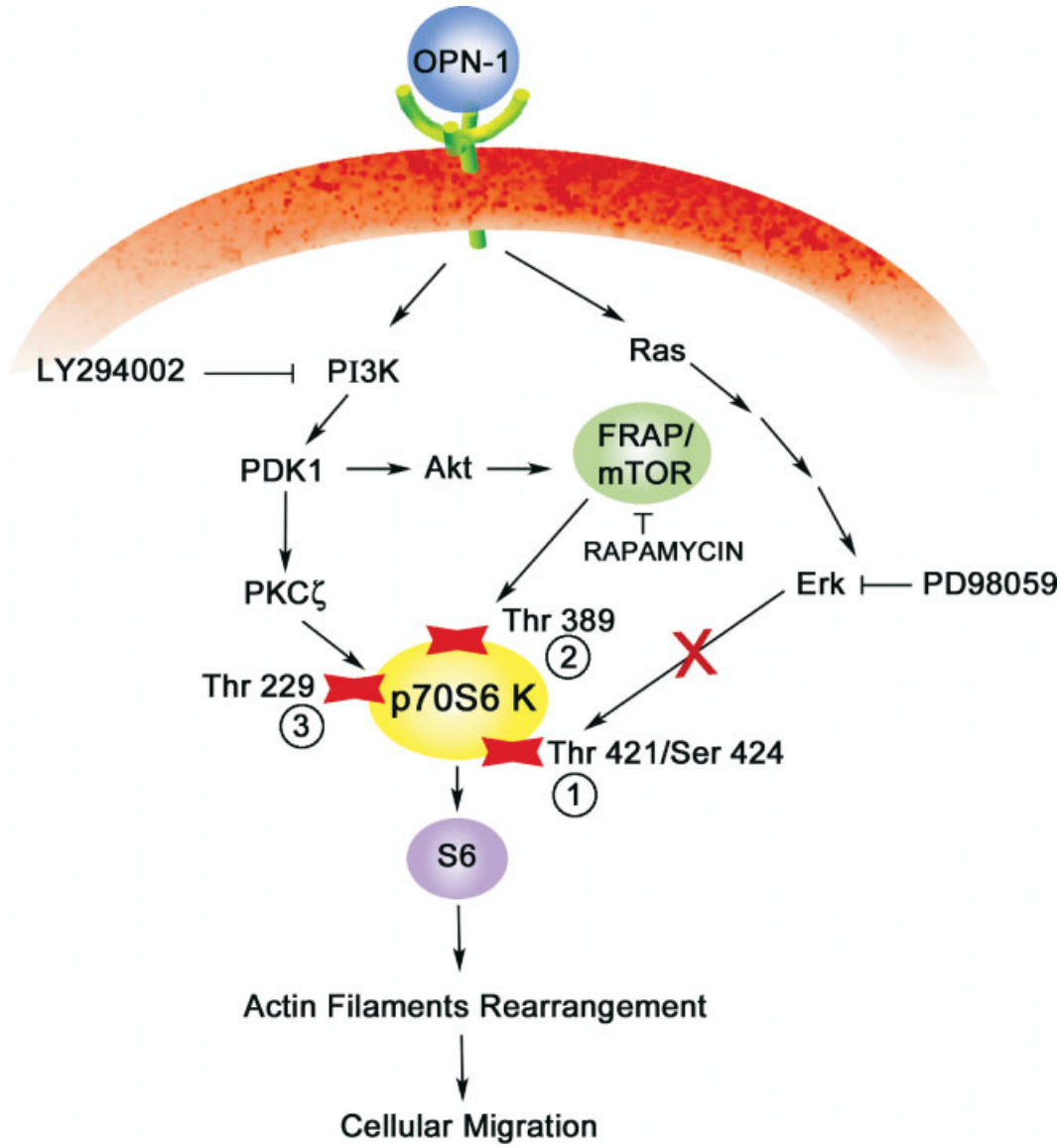


**Fig. 10.** Proposed signaling pathway involved in the activation of p70 S6 kinase by OPN-1 in JAR human choriocarcinoma cells. Upon binding of OPN-1 to its cell-surface specific receptor, PI3K is activated which in turn leads to the phosphorylation of a number of downstream kinases and eventually the phosphorylation of mTOR. The phosphorylation of p70 S6 kinase is achieved through the PI3K/mTOR pathway rather than the Ras/MAPK pathway (Erk in diagram). Once p70 S6 kinase is sequentially phosphorylated at both the Thr421/Ser424 and Thr389 sites, it is activated, phosphorylates ribosomal protein S6, and triggers the JAR cell migratory response to OPN-1.

previously unidentified pathway that is activated upon the exposure of trophoblastic cells to the highly phosphorylated form of OPN (OPN-1). This is the first study demonstrating that there is a preferential requirement for high-level phosphorylation of OPN by migratory JAR cells and that activation occurs through a rapamycin-sensitive signaling pathway involving p70 S6 kinase. Present studies are underway to identify the exact sites of phosphorylation of OPN that are sensitive to TRAP and to identify the cell surface receptor(s), which are involved in migration responses.

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