Hepatocyte growth factor (HGF) is important for cell proliferation, differentiation, and related activities. HGF acts through its receptor c-Met, which activates downstream signaling pathways. HGF binds to c-Met at the plasma membrane, where it is generally believed that c-Met signaling is initiated. Here we report that c-Met rapidly translocates to the nucleus upon stimulation with HGF. Ca\(^{2+}\) signals that are induced by HGF result from phosphatidylinositol 4,5-bisphosphate hydrolysis and inositol 1,4,5-trisphosphate formation within the nucleus rather than within the cytoplasm. Translocation of c-Met to the nucleus depends upon the adaptor protein Gab1 and importin \(\beta_1\), and formation of Ca\(^{2+}\) signals in turn depends upon this translocation. HGF may exert its particular effects on cells because it bypasses signaling pathways in the cytoplasm to directly activate signaling pathways in the nucleus.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—SkHep1 cells were cultured at 37 °C in 5% CO\(_2\) in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal bovine serum, 1 mm sodium pyruvate, 50 units/ml penicillin, and 50 g/ml streptomycin (Invitrogen).

**Immunoblots**—SkHep1 cell immunoblots were performed as described previously (19, 20). The NE-PER kit (Pierce) was used to prepare nuclear and cytosolic cell fractions (20). Briefly, cell membranes were disrupted to release cytoplasmic contents. Intact nuclei were recovered from the cytoplasmic extract by centrifugation, and then the nuclei were washed with PBS and lysed to yield the nuclear extract. Protease and phosphatase inhibitors (Sigma) were added to all buffers. Blots were visualized by enhanced chemiluminescence and quantitatively analyzed using a GS-700 imaging densitometer (Bio-Rad).

**Detection of PIP\(_2\)**—The phosphatidylinositol 4,5-bisphosphate mass strip kit was used for isolation and phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) detection (Echelon). SkHep1 cells were starved overnight in serum-free Dulbecco's modified Eagle's medium. Cells were used in aliquots of 5 \(\times\) 10\(^6\) cells/sample and incubated without or with HGF (100 ng/ml) for 4 min or arginine vasopressin (AVP) (100 nM) for 30 s, and then the medium was aspirated, and cellular material was precipitated with trichloroacetic acid (19, 20). The NE-PER kit (Pierce) was used to prepare nuclear and cytosolic cell fractions (20). Briefly, cell membranes were disrupted to release cytoplasmic contents.

**Detection of PIP\(_1\)**—The phosphatidylinositol 4-phosphate mass strip kit was used for isolation and phosphatidylinositol 4-phosphate (PIP\(_1\)) detection (Echelon). SkHep1 cells were starved overnight in serum-free Dulbecco's modified Eagle's medium. Cells were used in aliquots of 5 \(\times\) 10\(^6\) cells/sample and incubated without or with HGF (100 ng/ml) for 4 min or arginine vasopressin (AVP) (100 nM) for 30 s, and then the medium was aspirated, and cellular material was precipitated with trichloroacetic acid (19, 20). The NE-PER kit (Pierce) was used to prepare nuclear and cytosolic cell fractions (20). Briefly, cell membranes were disrupted to release cytoplasmic contents.

**Detection of PIP**—The phosphatidylinositol 3-phosphate mass strip kit was used for isolation and phosphatidylinositol 3-phosphate (PIP) detection (Echelon). SkHep1 cells were starved overnight in serum-free Dulbecco's modified Eagle's medium. Cells were used in aliquots of 5 \(\times\) 10\(^6\) cells/sample and incubated without or with HGF (100 ng/ml) for 4 min or arginine vasopressin (AVP) (100 nM) for 30 s, and then the medium was aspirated, and cellular material was precipitated with trichloroacetic acid (19, 20). The NE-PER kit (Pierce) was used to prepare nuclear and cytosolic cell fractions (20). Briefly, cell membranes were disrupted to release cytoplasmic contents.

**Detection of Inositol 1,4,5-trisphosphate (InsP\(_3\))**—The phosphatidylinositol 3-phosphate mass strip kit was used for isolation and phosphatidylinositol 3-phosphate (PIP) detection (Echelon). SkHep1 cells were starved overnight in serum-free Dulbecco's modified Eagle's medium. Cells were used in aliquots of 5 \(\times\) 10\(^6\) cells/sample and incubated without or with HGF (100 ng/ml) for 4 min or arginine vasopressin (AVP) (100 nM) for 30 s, and then the medium was aspirated, and cellular material was precipitated with trichloroacetic acid (19, 20). The NE-PER kit (Pierce) was used to prepare nuclear and cytosolic cell fractions (20). Briefly, cell membranes were disrupted to release cytoplasmic contents.

**Detection of Protein**—The phosphatidylinositol 3-phosphate mass strip kit was used for isolation and phosphatidylinositol 3-phosphate (PIP) detection (Echelon). SkHep1 cells were starved overnight in serum-free Dulbecco's modified Eagle's medium. Cells were used in aliquots of 5 \(\times\) 10\(^6\) cells/sample and incubated without or with HGF (100 ng/ml) for 4 min or arginine vasopressin (AVP) (100 nM) for 30 s, and then the medium was aspirated, and cellular material was precipitated with trichloroacetic acid (19, 20). The NE-PER kit (Pierce) was used to prepare nuclear and cytosolic cell fractions (20). Briefly, cell membranes were disrupted to release cytoplasmic contents.
tated by the immediate addition of 3 ml of ice-cold 0.5 M trichloroacetic acid. The NE-PER kit (Pierce) was used to prepare the nuclear and cell fraction, as described above (20). Briefly, cell membranes were disrupted to release cytoplasmic contents. Intact nuclei were recovered from the cytoplasmic extract by centrifugation, and then the nuclei were washed with PBS and precipitated with 3 ml of ice-cold 0.5 M trichloroacetic acid. Isolation of lipids was performed according to the manufacturer’s instructions and as described previously (21). The organic phase was collected into a clean tube and dried in a SpeedVac centrifuge. The pellet at this stage was faintly visible. The lipids were then resuspended by sonication in a cold water bath in 10 μl of CHCl₃:MeOH:H₂O (1:2:0.8) and spotted onto nitrocellulose membrane strips prespotted with phosphatidylinositol 4,5-bisphosphate standards, PIP controls, and space for spotting unknown samples for probing with anti-PIP₂ monoclonal antibody (Echelon) to specifically detect PIP₂. Blots were visualized by enhanced chemiluminescence and quantitatively analyzed using a GS-700 imaging densitometer (Bio-Rad).

**Assay for Cell Surface Protein by Biotinylation**—Cells were washed three times with 10 ml of PBS and subsequently incubated for 30 min at room temperature in 5 ml of PBS containing 1 mM sulfo-NHS-biotin (Pierce). Reactions were quenched for 10 min with 10 ml of PBS containing 100 μM glycine. Cells were stimulated with 100 ng/ml HGF (R&D Systems) for 4 min and lysed on ice for 10 min in 500 μl of NE-PER to prepare nuclear and cytosolic cell fractions (20). Streptavidin-agarose beads (Pierce) (50 μl of a 50:50 slurry) or anti-c-Met antibody were added to 500 μl of adjusted cell lysate, containing 200–500 μg of protein, and the suspension was rocked overnight at 4 °C. The samples were incubated with anti-c-Met antibody and 50 μl of agarose G for 2 h at room temperature. The beads were washed five times with 1 ml of PBS and analyzed by immunoblotting. Mouse monoclonal anti-c-Met antibody (Upstate Biotechnology) or streptavidin conjugated to horseradish peroxidase (Pierce) was used.

**Immunofluorescence**—Confocal immunofluorescence was performed as described (19, 20). Cells were triple-labeled with monoclonal antibodies against c-Met (Upstate Biotechnology) and polyclonal antibodies against Lamin B1 (AbCam) plus the nuclear stain TO-PRO-3 (Invitrogen). Images were obtained using a Zeiss LSM 510 confocal microscope using a ×63, 1.4 NA objective lens with excitation at 488 nm and observation at 505–550 nm for detecting Alexa Fluor 488, excitation at 543 nm and observation at 560–610 nm for detecting Alexa Fluor 555, and excitation at 633 nm and observation using LP650 nm to detect TO-PRO-3 (Invitrogen).

**InsP₃ Buffer Constructs**—The InsP₃ binding domain (residues 224–605) of the human type I InsP₃ receptor was tagged with monomeric red fluorescent protein (mRFP), and then the nuclear localization signal was subcloned to generate the nuclear InsP₃ buffer expression vector. The nuclear exclusion signal sequence derived from MKK1 was subcloned in the InsP₃ binding domain tagged with the mRFP construct to generate the cytoplasmic InsP₃ buffer expression vector, as described previously (22, 23).

**Transfection of siRNA**—SkHep1 cells were transfected using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen) with 50 nm siRNA for Gab1 (siRNA ID 1.927) (Ambion). The cells were incubated at 37 °C in an atmosphere of 5% CO₂, 95% O₂ for 48 h prior to use.

**Detection of Ca²⁺ Signals**—For Ca²⁺ imaging, cells were incubated with fluo-4/AM (6 μM) (Invitrogen) for 30 min at 37 °C, and then coverslips containing the cells were transferred to a custom-built perfusion chamber on the stage of a Zeiss LSM 510 confocal microscope, and fluo-4 fluorescence was monitored using a ×63, 1.4 NA objective lens at 1–5 frames/s. Changes in fluorescence were normalized by the initial fluorescence (F₀) and were expressed as (F/F₀) × 100% (19, 20). In selected experiments, cells were transfected for 48 h prior to study with targeted mRFP-InsP₃ buffer constructs using FuGENE 6 (Roche Applied Science).

**Statistical Analysis**—Significance of changes in treatment groups relative to controls were determined by Student’s t test. Data are represented as mean ± S.E.
c-Met Translocates to the Nucleus—Studies were performed in the SkHep1 liver cell line because of the well known effects of HGF in liver and because this liver cell line contains Ca²⁺/H₁₁₀₀₁ signage machinery in both the cytoplasm and the nucleus (20). As expected, c-Met was excluded from the nucleus of SkHep1 cells prior to stimulation with HGF (Fig. 1A). The phosphorylated, and hence active, form of c-Met accumulated in both the non-nuclear and the nuclear cell fraction of stimulated cells, reaching a peak in both compartments within 4 min (Fig. 1, A–C). The purity of nuclear preparations was confirmed using several nuclear and non-nuclear markers (Fig. 1D), although endoplasmic reticulum markers were not used because endoplasmic reticulum proteins can be found in the nucleus (20). Confocal immunofluorescence microscopy demonstrated that this receptor was at the plasma membrane or within the cyto-
plasm prior to stimulation and revealed that c-Met appeared both at the nuclear envelope and within the nuclear interior within 4 min of exposure to HGF (Fig. 2, A and B). In addition, c-Met that was biotinylated at the cell surface prior to stimulation with HGF could be recovered in the nucleus after cells were stimulated (Fig. 3). Together, these findings demonstrate that both total and activated c-Met rapidly translocates from the plasma membrane to the nucleus upon stimulation of cells with HGF.

FIGURE 5. c-Met generates InsP$_3$ in the nucleus rather than the cytoplasm. A, vasopressin-induced Ca$^{2+}$ signals are blocked by the cytosolic but not the nuclear InsP$_3$ buffer. Cells were loaded with fluo-4 and then stimulated with 100 nM AVP while examined by confocal microscopy. The red box indicates the region of interest in the nucleus, and the white box represents the region of interest in the cytoplasm that was used to monitor Ca$^{2+}$ signals. min., minimum; max., maximum. B, graphical representation of the nuclear and cytosolic Ca$^{2+}$ signal detected in a representative cell from each experimental group stimulated with vasopressin. The traces confirm that the cytosolic but not the nuclear InsP$_3$ buffer blocks all Ca$^{2+}$ signaling. C, HGF-induced Ca$^{2+}$ signals are blocked by the nuclear but not the cytosolic InsP$_3$ buffer. Cells were stimulated with HGF (100 ng/ml) while examined by confocal microscopy. The red box indicates the region of interest in the nucleus, and the white box represents the region of interest in the cytoplasm that was used to monitor Ca$^{2+}$ signals. D, graphical representation of the nuclear and cytosolic Ca$^{2+}$ signal detected in a representative cell from each experimental group stimulated with HGF. The tracings confirm that the nuclear but not the cytosolic InsP$_3$ buffer blocks all Ca$^{2+}$ signaling. E, summary of InsP$_3$ buffer studies confirms that AVP Ca$^{2+}$ signaling depends upon cytosolic InsP$_3$, whereas HGF Ca$^{2+}$ signaling depends upon nuclear InsP$_3$. Values are mean ± S.E. of the peak fluo-4 fluorescence attained during the observation period (expressed as % of baseline) and include the response from 10–15 cells in each experimental group (*, p < 0.05). F, cytoplasmic and nuclear Ca$^{2+}$ signals in representative cells examined at a rate of 83 ms/image. The vasopressin-induced Ca$^{2+}$ signal begins in the cytoplasm, whereas the HGF-induced signal begins in the nucleus. Note the expanded time scales; each agonist was added at t = 0.

c-Met Forms InsP$_3$ in the Nucleus—HGF stimulates cell proliferation, which has been linked to nuclear rather than cytoplasmic Ca$^{2+}$ signals (18), so we examined whether c-Met forms InsP$_3$ in the nucleus or cytoplasm. The nucleus is known to contain PLC and PIP$_2$ (24), which are necessary for the formation of InsP$_3$. The nucleus also contains InsP$_3$-gated Ca$^{2+}$ stores, within both the nuclear envelope (25–27) and the nucleoplasmic reticulum (20). To define whether c-Met forms InsP$_3$ in the cytoplasm or nucleus, we targeted the ligand binding domain (residues 224–605) of the InsP$_3$ receptor (InsP$_3$R) type 1 (22) to one or the other of these two compartments using a nuclear exclusion signal (Fig. 4A, NES) or nuclear localization signal (NLS) sequence, respectively, plus mRFP to verify localization (Fig. 4, A and B). RFP fluorescence was slightly greater in the construct expressed in the nucleus (Fig. 4C), perhaps because the construct was distributed in a slightly smaller compartment. This segment of InsP$_3$R1 specifically binds to InsP$_3$ with sufficient affinity to compete for binding to the native receptor (22). These targeted InsP$_3$ buffer constructs were
Ca\(^{2+}\) signaling was nearly abolished in cells expressing the cytoplasmic InsP\(_3\) buffer (Fig. 5, A, B, and E). These results show that vasopressin increases InsP\(_3\) only in the cytoplasm and that Ca\(^{2+}\) signals in both the cytoplasm and the nucleus result from this. Like vasopressin, HGF (100 ng/ml) increased Ca\(^{2+}\) in both the cytoplasm and the nucleus (Fig. 5, C–E). However, Ca\(^{2+}\) signals induced by HGF were nearly abolished in cells expressing the nuclear InsP\(_3\) buffer rather than the cytoplasmic buffer (Fig. 5, C–E). These results show that although phosphorylated c-Met is in both the nucleus and the cytoplasm (Fig. 1A), HGF increases InsP\(_3\) only in the nucleus, and Ca\(^{2+}\) signals throughout the cell result from this. Consistent with this, Ca\(^{2+}\) signaling detected in cells in which images were collected more rapidly (Fig. 5F) occasionally identified cells in which vasopressin-induced signals began in the cytoplasm, whereas HGF-induced signals began in the nucleus, similar to what has been observed previously (20). In other cells, Ca\(^{2+}\) signals appeared to begin simultaneously in both compartments, but vasopressin-induced signals never began in the nucleus, whereas HGF signals never began in the cytoplasm. To further demonstrate that HGF directly forms InsP\(_3\) within the nucleus, total and nuclear PIP\(_2\) hydrolysis was examined. Total cellular PIP\(_2\) was 17.8 \pm 1.9 pmol/5 \times 10^6 cells under control conditions. PIP\(_2\) was detected in the nucleus as well, although this represented less than 15\% of total cellular PIP\(_2\). Vasopressin was more effective than HGF in reducing total cellular PIP\(_2\), but HGF selectively and potently reduced PIP\(_2\) within the nucleus (Fig. 6, A and B). Vasopressin had minimal effect on nuclear PIP\(_2\) (n = 2, not shown). PLC-\(\gamma\) is thought to be respon-

pressed in the SkHep1 liver cell line, which express not only c-Met but also the G protein-coupled\(V_{1\alpha}\) vasopressin receptor (28). Stimulation of the\(V_{1\alpha}\) receptor was used as a cytoplasmic InsP\(_3\) control since G protein-coupled receptors activate PLC and form InsP\(_3\) at the plasma membrane (29). Vasopressin (100 nm) increased Ca\(^{2+}\) in both the cytoplasm and the nucleus, in control (non-transfected) cells as well as in cells transfected with the nuclear InsP\(_3\) buffer (Fig. 5, A, B, and E). However, expressed in the SkHep1 liver cell line, which express not only c-Met but also the G protein-coupled\(V_{1\alpha}\) vasopressin receptor (28). Stimulation of the\(V_{1\alpha}\) receptor was used as a cytoplasmic InsP\(_3\) control since G protein-coupled receptors activate PLC and form InsP\(_3\) at the plasma membrane (29). Vasopressin (100 nm) increased Ca\(^{2+}\) in both the cytoplasm and the nucleus, in control (non-transfected) cells as well as in cells transfected with the nuclear InsP\(_3\) buffer (Fig. 5, A, B, and E). However, expressed in the SkHep1 liver cell line, which express not only c-Met but also the G protein-coupled\(V_{1\alpha}\) vasopressin receptor (28). Stimulation of the\(V_{1\alpha}\) receptor was used as a cytoplasmic InsP\(_3\) control since G protein-coupled receptors activate PLC and form InsP\(_3\) at the plasma membrane (29). Vasopressin (100 nm) increased Ca\(^{2+}\) in both the cytoplasm and the nucleus, in control (non-transfected) cells as well as in cells transfected with the nuclear InsP\(_3\) buffer (Fig. 5, A, B, and E). However,
sible for HGF-induced hydrolysis of PIP_2, and both immunoblot (Fig. 6C) and confocal immunofluorescence (not shown) demonstrated that this isoform of PLC is in the nucleus as well as in the cytoplasm. Together, these findings identify c-Met as an endogenous factor that selectively activates the nuclear machinery responsible for hydrolyzing PIP_2 and thus generating InsP_3 and Ca^{2+} signals within the nucleus.

**c-Met Must Translocate to the Nucleus to Induce Ca^{2+} Signals**—To determine whether c-Met must go to the nucleus to initiate nuclear Ca^{2+} signaling, the role of Gab1 and importin-β1 was examined. Gab1 is an adaptor protein that contains a nuclear localization sequence (30) and also binds to c-Met and to other RTKs (31). Transport of proteins through the nuclear pore complex typically involves importins α/β and exportins (32). Specifically, importin-α binds to the classical lysine-rich nuclear localization signal in the cargo, and importin-β interacts with the importin-α/cargo complex to guide it through the nuclear pore (32). Immunoblots of cell fractions isolated at serial time points following HGF stimulation demonstrated that HGF induced translocation of Gab1 to the nucleus (Fig. 7). To determine whether Gab1 or importin-β1 are necessary for the translocation of c-Met to the nucleus, siRNAs were developed that reduced Gab1 expression by 83 ± 6% (Fig. 8A) and importin expression by 90 ± 7% (Fig. 8C) in SkHep1 cells, relative to non-transfected and scrambled siRNA-transfected controls. Immunoblots of nuclear and non-nuclear cell fractions demonstrated that treatment of cells with Gab1 and importin siRNAs reduced the translocation of c-Met to the nucleus by 48 ± 6% (Fig. 8B) and 55% ± 2% (Fig. 8D), respectively. These results suggest that Gab1 and importin each play a role in c-Met translocation. Finally, HGF-induced Ca^{2+} signals in both the nucleus and the cytoplasm were eliminated in transfected with either Gab1 or importin siRNA (Fig. 9, A–C). This action of these siRNAs did not reflect a nonspecific inhibition of Ca^{2+} signaling pathways because vasopressin-induced Ca^{2+} signals were not inhibited (Fig. 9B). These findings demonstrate that Gab1- and importin-mediated translocation of c-Met to the nucleus is necessary to initiate nuclear Ca^{2+} signaling.

**DISCUSSION**

Our results suggest that Ca^{2+} signaling induced by RTKs such as c-Met occurs through direct formation of InsP_3 in the nucleus, which differs fundamentally from Ca^{2+} signaling induced by G protein-coupled receptors such as the V_{1a} receptor (Fig. 10). The cell nucleus contains the machinery necessary for locally generating InsP_3 and InsP_3-dependent Ca^{2+} signals, including PIP_2, PLC (33), and InsP_3-dependent Ca^{2+} stores (20, 27). Several lines of evidence suggest that growth factors (33) and integrins (34) may activate nuclear isoforms of PLC, and the current work demonstrates the functional significance of this by showing that HGF, via c-Met, hydrolyzes PIP_2 to form InsP_3 and increase free Ca^{2+} within the nucleus. It is now appreciated that Ca^{2+} signals within the nucleus exert a range of cellular effects that cannot be induced by cytoplasmic Ca^{2+} signals, including stimulation of cell proliferation (18), activation of transcription factors such as CAMP-response element-binding protein (35) and Elk-1 (23), and activation of kinases such as protein kinase C (20) and calmodulin kinase IV (36).

The observation that c-Met induces InsP_3 formation directly within the nucleus, and must do so to generate Ca^{2+} signals, provides insight as to how Ca^{2+}-dependent events within the nucleus are controlled. The current findings also provide evidence that activation of c-Met generates little or no InsP_3 in the cytoplasm, in contrast to what is widely believed (5–7). It is not yet clear why phosphorylated c-Met activates PLC only in the nucleus, and not at the plasma membrane, before the receptor translocates to the nucleus. One possible explanation is that the proper isoform of PLC does not associate with c-Met until the receptor reaches the nucleus, perhaps because the internalization process of c-Met at the plasma membrane makes it inaccessible to cytoplasmic PLC or because adaptor proteins in the
A number of RTKs have been found in the nucleus, including receptors for insulin, epidermal growth factor receptor, and fibroblast growth factor (8–10). These nuclear RTKs have been shown to act as transcription factors (9, 38). However, the mechanism by which RTKs travel from the cell surface to the nucleus is unknown. RTKs generally are internalized by clathrin-dependent endocytosis (39). Desensitization of both the epidermal growth factor receptor and the HGF receptor occurs by a common mechanism in which internalization is dependent upon clathrin but also depends upon association with a complex consisting of Cbl, CIN85, and endophilin. This complex enables Cbl to induce ubiquitination and then degradation of these receptors (40, 41). It is unclear whether transport of RTKs to the nucleus begins with this same mechanism of internalization because disruption of this tripartite protein complex prevents internalization of the HGF receptor yet enhances rather than impairs the biological response to HGF (40). Additional questions include how c-Met enters the nucleus, the topology of c-Met within the nucleus, and whether HGF remains bound to nuclear c-Met.

Ca²⁺ signals can consist of complex spatial patterns, including gradients and waves, and the subcellular distribution of InsP₃Rs helps shape these patterns. For example, presynaptic increases in Ca²⁺ regulate neurotransmitter release (39), and local increases in Ca²⁺ also regulate extension of neural growth cones (42). Accessory proteins such as Homer and protein 4.1N are thought to localize InsP₃Rs to these regions in neurons (43, 44), which may in turn permit these highly localized Ca²⁺-mediated events to occur. Similarly, apical increases in Ca²⁺ regulate fluid and electrolyte secretion (45) and exocytosis (46) in polarized epithelia, and these localized Ca²⁺ signals result from the local accumulation of InsP₃Rs in lipid rafts (47, 48). Furthermore, apoptosis can be induced by transmission of Ca²⁺ signals from InsP₃Rs to mitochondria (49), and physical connections between these two structures may facilitate this (37). Finally,
gene transcription (23) and cell growth (18) are regulated by increases in Ca\(^{2+}\) in the nucleus, which in turn can be induced by release of Ca\(^{2+}\) from intranuclear InsP\(_3\)R isoforms (20). Moreover, there are differences in the types of InsP\(_3\)R isoforms found in the nucleus in different cell types, and this variability can affect the sensitivity of the nucleus to InsP\(_3\) (19). By demonstrating that growth factors can selectively increase InsP\(_3\) within the nucleus, the current findings reveal an even more sophisticated level of regulation in which nuclear InsP\(_3\)Rs can be activated not by virtue of their subcellular distribution but rather as a result of local, subcellular production of InsP\(_3\).