0013-7227/97/$03.00/0
Endocrinology

Vol. 138, No. 4
Printed in U.S.A.

NUCLEAR TRANSPORT OF INSULIN-LIKE GROWTH FACTOR-I AND INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN-3 IN OPOSSUM KIDNEY CELLS.

Wenlu Li*, Janet Fawcett #, H. Ramon Widmer*, Paul J. Fielder*, Ralph Rabkin # and Gilbert-André Keller*
*Department of Pharmacokinetics and Metabolism, Genentech, Inc., South San Francisco, CA 94080
#Department of Medicine, Stanford University and Veteran Affairs Medical Center, Palo Alto, CA 94304.

ABSTRACT

When added to cultured opossum kidney cells, IGF-I is internalized and transported to distinct intracellular compartments that depend on the cell location within the monolayer. In resting cells away from the periphery of the monolayer, IGF-I is internalized by a clathrin coated pit pathway and delivered to the endosomal compartment. In contrast, cells growing at the edges of a monolayer or an experimental wound internalize IGF-I by an alternative route which rapidly delivers IGF-I to the nucleus. Similarly to IGF-I, IGFBP-3 is also internalized and accumulates in the endosomal compartment in resting cells whereas it is targeted to the nucleus in proliferating cells. IGFBP-3, which contains a putative nuclear targeting signal, may act as a carrier for IGF-I nuclear transport. The transport of IGF-I and IGFBP-3 to two different compartments may influence their biological activity.

Most of the biological effects of insulin-like growth factor (IGF-I) are thought to be mediated through the Type I IGF receptor, a membrane bound glycoprotein involved in signaling of cell growth and metabolism. IGF-I also binds with high affinity to a family of six IGF-binding proteins (IGFBPs) which regulate its bioavailability and modulate its actions (1). Many growth factors interact with their receptors at the cell surface, leading to receptor autophosphorylation and production of multiple intermediate messengers. Rapid internalization and transport via a coated-vesicle pathway to the lysosomes where they are degraded are common features of most peptide growth factors (2). Over the last decade, several polypeptide hormones and growth factors including IGF-I have been found to internalize and translocate to the nucleus in target cells. In the embryonic chicken lens, IGF-I accumulates in the nuclei of epithelial cells but not in fiber cells. This observation suggests that the transport of IGF-I to different subcellular compartments may be related to the state of differentiation of the cells (3). Putative nuclear localization signals (NLS) have been identified in insulin like growth factor binding protein-3 (IGFBP-3) and IGFBP-5 sequences, but no NLS has been found in IGF-I itself (4). Early studies have shown that cells within a monolayer are in a resting state. However, when the confluent culture is wounded, new growth occurs at the margin of the wound (5, 6). In this study, we show that, in resting cells, IGF-I and IGFBP-3 both internalize and localize to the endosomal compartment whereas, in proliferating cells at the edge of the wound or at the periphery of the culture, IGF-I and IGFBP-3 accumulated in the nucleus after transiting through the cytoplasm.

MATERIAL AND METHODS

Cell cultures. OK cells, a cell line with features of proximal tubule epithelium derived from opossum kidney, were cultured on glass coverslips for fluorescence microscopy and on Petri dishes or on filters (GIBE) for electron microscope autoradiography (7).

Received: 09/17/96

Fluorescent IGF-I and IGFBP-3 analogs. Human recombinant IGF-I and IGFBP-3 were expressed in *E. coli* and CHO cells, respectively (8). IGF-I, des-(1-3) IGF-I and IGFBP-3 were conjugated with the fluoroprobes indocarbo-cyanine (Cy3) and dichlorotrainylfluorescein DiHCl (DTAF) (Organic Research). Fluorescent IGF-I retained the ability to bind to and phosphorylate the type I receptor. Fluorescent IGFBP-3 bound IGF-I with a slightly reduced affinity.

Internalization experiments. OK cells incubated in medium without serum for 3 to 18 hr were treated with 1-5 μg/ml of fluorescent IGF-I, des-(1-3) IGF-I or/and IGFBP-3 for 0 to 120 min at 37°C. Experimental wounds were created by scratching a confluent monolayer with a scalpel blade (5, 6) before treatment. Cells were then fixed in neutral formaldehyde, mounted in buffered-glycerol and observed in a Molecular Dynamics Confocal Microscope 2001. In control experiments, cells were incubated with Cy3 or DTAF alone, or with Cy3-rhDNase, a protein with a MW similar to IGFBP-3.

Electron Microscopy. OK cells were incubated with ^{125}I-IGF-I at 37°C for 0 to 120 min, fixed and processed for autoradiography as described (9).

RESULTS.

Fluorescent conjugated IGF-I and IGFBP-3 were used to follow the uptake and intracellular transport of these molecules rather than conventional immunocytochemistry since many cell lines produce IGF-I and IGFBPs. When OK cells were incubated with Cy3-IGF-I, two different fluorescent patterns were observed. In proliferating cells growing from the confluent region of the monolayer or at the edge of a wound, IGF-I was detected in the nucleus as early as 10 min after treatment (Fig. 1A and B). In contrast, in the resting cells away from the periphery of the monolayer, Cy3-IGF-I was taken up and sequestered in vesicular organelles (Fig. 1A inset). No fluorescence was observed in the nucleus in the quiescent cells.

The distribution of IGF-I in two subcellular compartments was further examined by electron

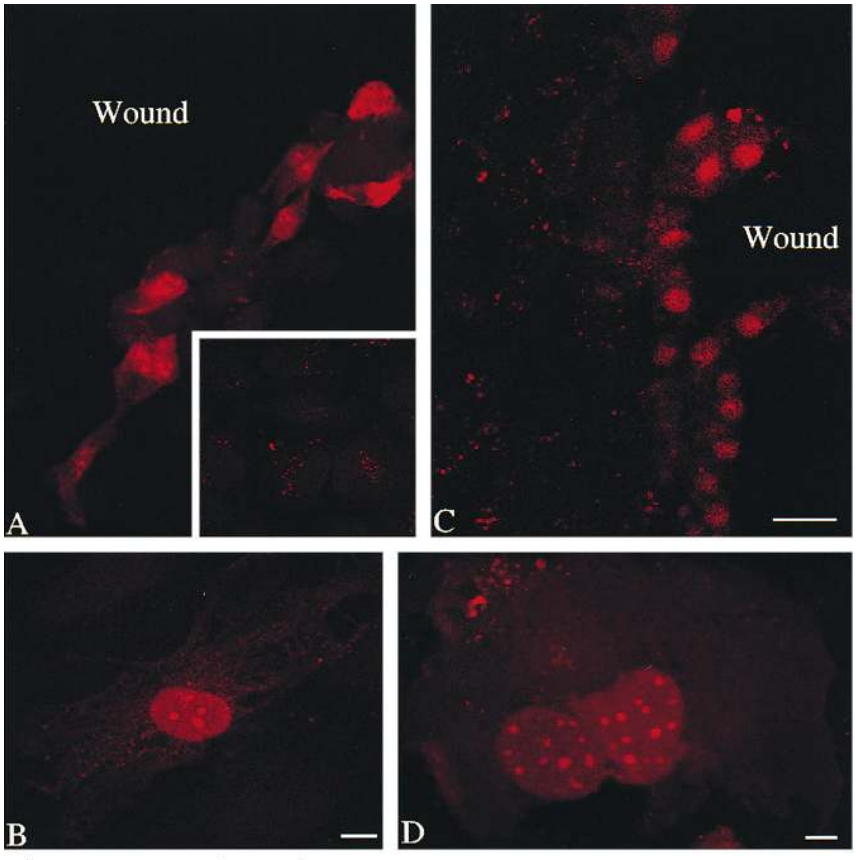

Fig. 1. Localization of IGF-I and IGFBP-3 analyzed by fluorescence confocal microscopy shows that Cy3-IGF-I (A, B) and Cy3-IGFBP-3 (C, D) are internalized and transported to the nucleus in proliferating cells at the edge of the wound. In cells away from the wound, IGF-I (A, inset) and IGFBP-3 accumulate in vesicular structures in the cytoplasm. B and D are high magnifications demonstrating nuclear accumulation of IGF-I (B) and IGFBP-3 (D) in cells at the periphery of the monolayers.
Bar in B = 50 μm.
Bars in C and D = 10 μm.

microscopy autoradiography. In proliferating cells, autoradiographic silver grains were first detected at the plasma membrane (Fig. 2A). Within 10 min numerous silver grains were observed in the cytosol without apparent association with coated pits, vesicles or membrane bound organelles, and over the nucleus (Fig. 2B and C). In contrast, in the quiescent cells, ^{125}I-IGF-I was internalized via coated pits at both the apical and basolateral membranes (Fig. 2D) and accumulated in the organelles of the endocytotic pathway (Fig. 2 E and F). The nuclei of the quiescent cells were free of silver grains.

As IGFBP-3 contains a NLS we hypothesized that IGFBP-3 may act as a carrier for IGF-I nuclear transport. We consequently treated cells with Cy3-IGFBP-3 without addition of IGF-I. Consistent with the presence of a NLS in its sequence, IGFBP-3 accumulated in the nuclei in the cells at the edge of the wound (Fig. 1 C and D). In resting cells, however, IGFBP-3 was located in the endosomal compartment (Fig. 1 C). When the monolayer was simultaneously treated with fluorescent IGF-I and IGFBP-3, both molecules accumulated in an apparently synchronous manner in the nuclei of proliferating cells (Fig. 3A-C) but in vesicular structures that correspond to the endosomal compartment in the resting cells (Fig. 3D-F). Cy3-des-(1-3) IGF-I, an analog of IGF-I that has reduced affinity for IGFBP-3, was not transported to the nucleus of proliferating cells even in the presence of IGFBP-3. No fluorescence was detected within the cells incubated with free Cy3 or DTAF. The control molecule Cy3-DNase internalized and accumulated in the endosomes of both resting and proliferating cells but not in their nuclei (not shown).

DISCUSSION.

IGF-I and IGFBP-3 follow different intracellular pathways according to the position of the cells in the monolayer. In quiescent cells away from the periphery of the monolayer, IGF-I and IGFBP-3 are internalized and enter the endosomal compartment. In these cells, IGF-I internalization is consistent with a receptor-mediated pathway (3,7) but it remains to be determined whether the internalization of IGFBP-3 is also via a receptor-mediated process involving the IGFBP-3 receptor (10). In contrast to quiescent cells, IGF-I and IGFBP-3 appear to cross the plasma membrane in proliferating cells by an alternate pathway which leads to nuclear accumulation. The mode of IGF-I and IGFBP-3 internalization in proliferating cells is unclear although several mechanisms can be considered. First, it is conceivable

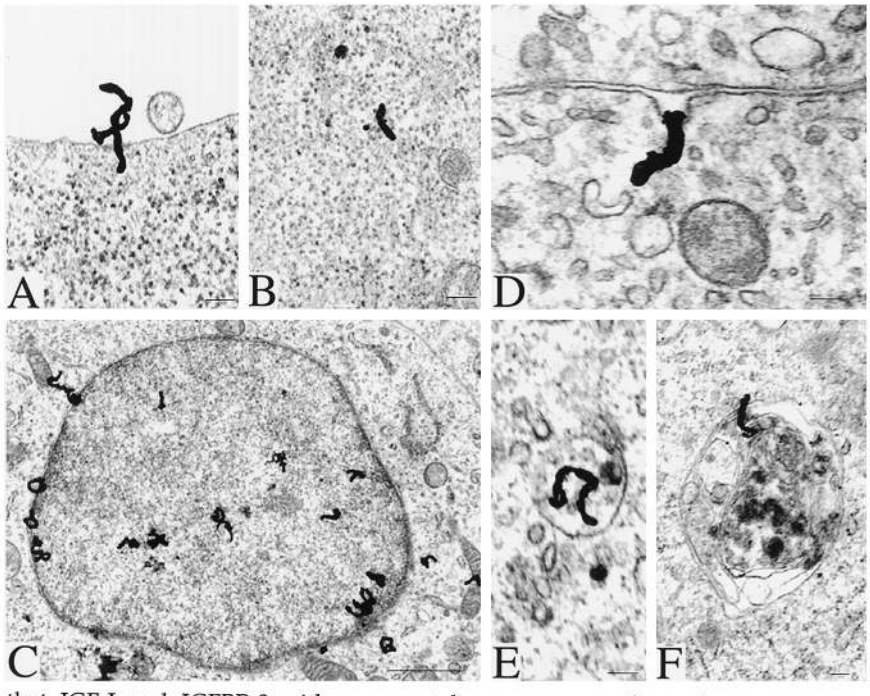

Fig. 2. Subcellular localization of ^{125}I-IGF-I in OK cells. In proliferating cells (A, B and C), the autoradiographic grains are found over the plasma membrane (A) in the cytosol (B) with no apparent association with vesicular structure. Within 10 min after treatment, numerous silver grains are visualized over the nucleus (C) while in resting cells (D, E and F) grains are observed associated with a coated pit at the basolateral membrane (D) and late endosomes (E and F). Bars in A, B, D, E and F= 0.1 μm; Bar in C=1 μm.

that IGF-I and IGFBP-3, either separately or as a complex, internalize and are released from early endosomes into the cytosol. Similar mechanisms have been proposed to explain the translocation of endocytosed growth hormone and insulin into cytosol and the nucleus (11, 12). Against this is the finding that no IGF-I was observed in the early endosomes of proliferating cells. Furthermore, Western ligand blot indicated that OK cells produce IGFBPs, including a 42 KD binding protein of the same MW as IGFBP-3 (data not shown). Accordingly, we speculate that IGF-I and IGFBP-3, either added or produced by OK cells, form a complex that crosses the plasma membrane by an internalization pathway independent of the endocytosis via coated pits. Complex formation is likely to take place in the medium as IGF-I binds with higher affinity to IGFBP-3 than to its receptor (1).

Different models have recently been proposed that may explain the rapid translocation of exogenous proteins through the plasma membrane by mechanisms which require the presence of yet unidentified cell surface unfoldases and protein transporters (13, 14). We have reported that heregulin-β1, a protein that activates the ErbB receptor tyrosine kinases family, is rapidly internalized and translocates to the nucleus of breast cancer cells. Heregulin, which contains a putative NLS, also crosses the plasma membrane with no apparent involvement of the coated vesicle or caveolae pathway (9). Thus, translocation across the plasma membrane into the cytosol may be an early step in a pathway common to growth factors that traffic to the nucleus.

At least two mechanisms could account for the accumulation of IGF-I and IGFBP-3 in the nucleus. One is the separate internalization and nuclear transport of the two molecules. Once internalized and released in the cytosol, a small molecule such as IGF-I could enter the nucleus through the nuclear pores by free diffusion whereas IGFBP-3 would be selectively targeted to the nucleus by its NLS. We favor, however, an alternative mechanism which involves the cotransport of IGF-I and IGFBP-3 as a complex. As IGF-I, which does not contain a NLS, is translocated to the nucleus with kinetics similar as IGFBP-3, we speculate that IGFBP-3 acts as a carrier to which IGF-I binds, forming an IGF/IGFBP-3 complex that crosses the cell membrane. Once in the cytosol, the IGF/IGFBP-3 complex would be selectively targeted to the nucleus by the IGFBP-3 NLS. The fact that des(1-3)-IGF-I, even with added IGFBP-3, is not transported to the nucleus indicates that IGF-I may depend on IGFBP-3 for nuclear localization.

IGF-I and IGFBP-3 appear to be imported in the nucleus as intact molecules since most of IGF-I was still TCA precipitable 20 min after treatment of OK cells (7). Furthermore, no shift in the migration of IGF-I and IGFBP-3 could be detected in Western blots of nuclear fractions (not shown). These results raise intriguing questions regarding IGF-I and IGFBP-3 cellular functions in the nuclei of proliferating cells: First, is nuclear IGF-I able to trigger biological responses? Nuclear accumulation is critical for factors such as the

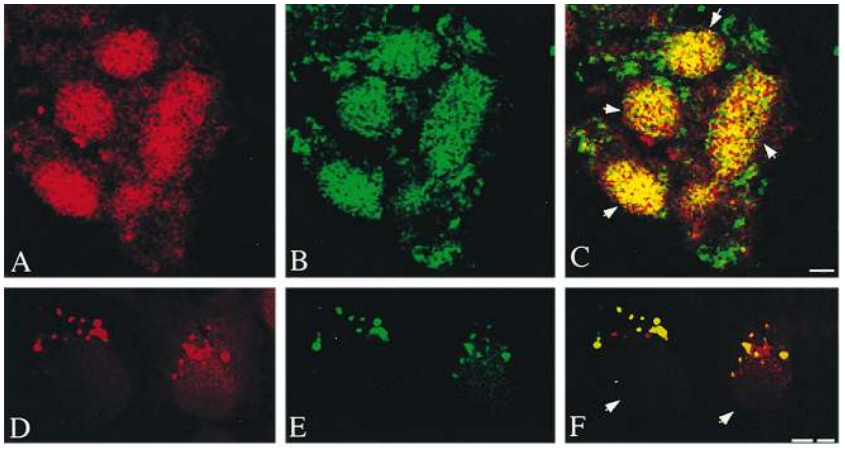

Fig. 3. Colocalization of Cy3-IGF-I and DTAF-IGFBP-3 by confocal microscopy. In cells facing the wound, IGF-I (A) and IGFBP-3 (B) accumulate in the nuclei. However, in cells away from the wound, IGF-I (D) and IGFBP-3 (E) localize to vesicular organelles in cytoplasm. C and F are combined images of A, B and C, D, respectively. Colocalization of IGF-I and IGFBP-3 appears in yellow. Arrows in C and F indicate the position of the nuclei. Bar = 10 μm.

Schwannoma-derived growth factor (15) and prolactin (16) as mutants defective in NLS are unable to initiate mitotic activity. Therefore, in addition to controlling gene transcription by signaling pathways (17, 18), it is conceivable that IGF-I acts as its own messenger, perhaps through complex interactions with other nuclear proteins (19, 20). It is also provoking to consider that nuclear IGFBP-3 may have specific actions independent of IGF-I. This may be relevant to recent studies showing that overexpression of IGFBP-3 has an inhibitory effect on cell growth which does not involve IGF-I binding to, or signal transduction via, the IGF receptor (21). Nuclear interactions between IGF-I, IGFBP-3 and other nuclear molecules may thus provide an additional control of cell growth and differentiation that is independent of the IGF-I receptor-kinase activation.

Acknowledgment: This study was funded by Genentech Inc. and the Veterans Administration.

REFERENCES

1. **J. I. Jones and D. R. Clemmons**, 1995 Insulin-like growth factors and their binding proteins: biological actions. Endocrine Reviews, 16: 3-34
2. **G. Carpenter**, 1987 Receptor for epidermal growth factor and other polypeptide mitogens. Annu. Rev. Biochem., 56: 881-914
3. **A. Peralta-Soler, J. Alemany, R. M. Smith, F. d. Pablo and L. Jarett**, 1990 The state of differentiation of embryonic chicken lens cells determines insulin-like growth factor internalization. Endocrinology, 127: 595-603
4. **R. T. Radulescu**, 1994 Nuclear localization signal in insulin-like growth factor-binding protein type 3. Trends-Biochem-Sci., 19: 278
5. **G. J. Todaro, G. Lazar and H. Green**, 1965 The inhibition of cell division in a contact-inhibited mammalian cell line. J Cell Comp. Physiol., 66: 325-334
6. **R. Dulbecco**, 1970 Topoinhibition and serum requirement of transformed and untransformed cells. Nature, 227: 802-806
7. **J. Fawcett and R. Rabkin**, 1996 The processing of insulin-like growth factor-I (IGF-I) by a cultured kidney cell line is altered by IGF-I binding protein-3. Endocrinology, 136: 1340-1347
8. **W. I. Wood, G. Cachanes, W. G. Henzel, J. A. Winslow, S. A. Spencer, R. Hellmass, J. L. Martin and M. C. Baxter**, 1988 Cloning and expression of growth hormone dependent insulin like growth factor protein binding. Molecular Endocrinology, 2: 1176-1185
9. **W. Li, J. W. Park, A. Nuijens, M. X. Sliwkowski and G-A. Keller**, 1996 Heregulin is rapidly translocated to the nucleus and its transport is correlated with c-myc induction in breast cancer cells. Oncogene, 12: 2473-2477
10. **Y. Oh, H. L. Muller, H. Pham and R. G. Rosenfeld**, 1993 Demonstration of receptors for insulin-like growth factor binding protein-3 on Hs578T human breast cancer cells. J. Biol. Chem., 268: 26045-26048
11. **P. E. Lobie, H. Mertani, G. Morel, O. Morales-Bustos, G. Norstedt and M. J. Waters**, 1994 Receptor-mediated nuclear translocation of growth hormone. J. Biol. Chem., 269: 21330-21339
12. **N. Shah, S. Zhang, S. Harada, R. M. Smith and L. Jarett**, 1995 Electron microscopic visualization of insulin translocation into the cytoplasm and nuclei of intact H35 hepatoma cells using covalently linked nanogold-insulin. Endocrinology, 136: 2825-2935
13. **A. Rubartelli and R. Sitia**, 1995 Entry of exogenous polypeptides into the nucleus of living cells: facts and speculations. Trends in Cell Biology, 5: 409-412
14. **F. Perez, A. Joliot, E. Bloch-Gallego, A. Zahraoui, A. Triller and A. Prochiantz**, 1992 Antennapedia homeobox as a signal for the cellular internalization and nuclear addressing of a small exogenous peptide. J. Cell Sci., 102: 717-722
15. **H. Kimura**, 1993 Schwannoma-derived growth factor must be transported into the nucleus to exert its mitogenic activity. Proc. Natl. Acad. Sci. USA, 90: 2165-2169
16. **C. V. Clevenger, S. W. Altman and M. B. Prystiwsky**, 1991 Requirement of nuclear prolactin for interleukin-2-stimulated proliferation of T lymphocytes. Science, 253: 77-79
17. **S. A. Rosenzweig, B. S. Oemar, N. M. Law, U. T. Shankavaram and B. S. Miller**, 1993 Insulin-like-growth factor 1 receptor signal transduction to the nucleus. Adv. Exp. Med. Biol., 343: 159-168
18. **B. H. Jhun, T. Haruta;, J. L. Meinkoth, W. Leitner, B. Draznin, A. R. Saltiel, L. Pang, T. Sasaoka and J. M. Olefsky**, 1995 Signal transduction pathways leading to insulin-induced early gene induction. Biochemistry, 34: 7996-8004
19. **R. T. Radulescu and C. M. Wendtner**, 1992 Proposed interaction between insulin and retinoblastoma protein. J. Mol. Recognit., 5: 132-137
20. **R. T. Radulescu and C. M. Wendtmer**, 1993 Hormone and growth factor subunits: a novel perception of cell growth regulation. J. of Endocrinol., 139: 1-7
21. **B. Valentinis, A. Bhala, T. DeAngelis, R. Baserga and P. Cohen**, 1995 The human Insulin Growth Factor (IGF) Binding Protein-3 Inhibits the growth of fibroblasts with targeted disruption of the IGF-I receptor gene. Molecular Endocrinology, 9: 361-367