An Analogue of IGF-I

A Potent Substitute for Insulin in Serum-Free Manufacture of Biologics By CHO Cells

Catherine Yandell, Jemma Lawson, Ivor Butler, Belinda Wade, Antony Sheehan, Sally Grosvenor, Chris Goddard, and Tony Simula

Chinese hamster ovary (CHO) cells are widely used to manufacture recombinant proteins for the biopharmaceutical industry. Although serum is often used for the propagation of CHO cell lines, there are compelling reasons for companies to develop manufacturing processes that do not include animal-derived products. For example, regulatory agencies are concerned about the potential to contaminate recombinant human therapeutics with adventitious agents that may be present in products derived from animals. In addition, technical issues such as batch-to-batch variability of commercial bovine serum, can make it difficult for manufacturers to maintain a satisfactory level of product consistency.

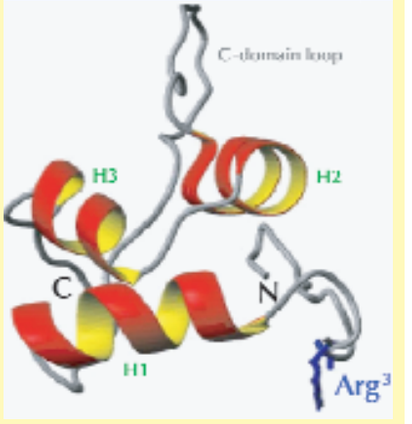
Traditionally the growth of mammalian cells in the absence of serum has required media supplementation with various growth factors, including insulin. Although insulin was originally purified from bovine and porcine pancreatic sources, recombinant forms have become available in the past few years. Insulin supports the serum-free (SF) growth of many cell types used in the biopharmaceutical industry only when added at high, nonphysiological concentrations of 2–10 mg/L (**1, 2**).

When serum-dependent CHO cells are adapted to grow as suspension cultures in protein-free or serum-free media (SFM), they are more susceptible to stresses that cause cell death (**2**). Under bioreactor conditions, the most significant mechanism driving loss of cell viability is an increase in apoptosis, programmed cell death (**3**). Using various cell lines, a number of approaches have been successful in preventing or delaying the onset of apoptosis (**2, 4**), including the deliberate overexpression of apoptosis-suppressor genes such as Bcl-2 and Bag-1 (**5, 6**) or the active inhibition of proapoptotic proteins such as caspase 3 (**7**). Those studies illustrate that apoptosis can have a marked influence on the performance of cells in a bioreactor.

How Does Insulin Work? Insulin and insulin-like growth factor (IGF)-I are closely related in amino acid sequence and tertiary structure. The receptors for IGF-I (IGF-IR) and insulin (IR) share extensive homology. In addition to binding their own receptors with high affinity, IGF-I and insulin are able to bind and activate each others' receptors with low affinity (**8**), requiring higher concentrations of ligand to achieve a similar level of receptor occupancy.

Several observations have led to the widespread belief that the cell culture potency of insulin can be attributed to signaling through the IGF-IR instead of its own receptor. CHO and most other cell lines have very few insulin receptors on their surfaces (Table 1). Insulin is required at supraphysiological concentrations to support the growth of most cell types, including CHO cells, under SF conditions (**11**). At concentrations used in cell culture (2–10 mg/L), insulin competes with

Solution structure of Long™R³IGF-I.



PRODUCT FOCUS: BIOLOGICS

PROCESS FOCUS: MEDIA DEVELOPMENT FOR MAMMALIAN CELLS (PRODUCTION)

WHO SHOULD READ: PROCESS DEVELOPMENT, MANUFACTURING

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LEVEL: INTERMEDIATE

IGF-I for binding to the IGF-IR. Furthermore, such a high concentration of insulin is much greater than would be required to induce a response from the IR (**11**). So it is unlikely that the effects of insulin in cell culture are mediated solely by insulin receptors on CHO cells. IR and IGF-IR share many downstream signaling pathways such as phosphoinositol 3 kinase (PI3K)/Akt and ras/raf/mitogen activated protein kinase (MAPK) (**12**). However, insulin is regarded as a major regulator of carbohydrate, protein, and lipid metabolism in cells (**13**), whereas IGFs play an important role in cell growth and survival (**14**). In particular, activation of the IGF-IR has been shown to mediate potent antiapoptotic actions (**15, 16**).

A More Potent Growth Factor: If activation of the IGF-IR is the mechanism by which insulin is acting in SF cell culture, then a more effective growth factor would be IGF-I (or IGF-II), which can activate IGF-IR at much lower concentrations than insulin can. The IGF-I analog manufactured by GroPep Limited, Long^{TM}R^{3}IGF-I, was specifically engineered for cell culture use and to support the survival and proliferation of mammalian CHO cells in SFM for large-scale commercial manufacture of recombinant human biopharmaceuticals. As shown in Figure 1, Long^{TM}R^{3}IGF-I outperforms insulin in supporting cell viability and protein production at 200-fold reduced concentrations in SFM (**11, 17**).

Long^{TM}R^{3}IGF-I has a distinct biological advantage over native IGF-I and IGF-II. In addition to its 13–amino-acid N-terminal extension, Long^{TM}R^{3}IGF-I was derived from native IGF-I by replacing the third amino acid of the mature peptide, glutamic acidate, with an arginine (**18**). This substitution gives Long^{TM}R^{3}IGF-I a >100-fold reduced affinity for IGF-binding proteins (IGFBPs) without compromising its biological potency (**18**). IGFBPs make up a group of six related proteins whose major physiological role is to control the availability of IGF-I and IGF-II binding to the IGF-IR (**19**). Most cell lines secrete certain forms of IGFBP. For example, CHO cells constitutively secrete IGFBP-4, and IGFBP-3 in response to native IGF-I (**20**), limiting the bioavailability of native IGF-I in SFM. By contrast, the bioavailability and hence potency of Long^{TM}R^{3}IGF-I is uncompromised no matter the levels of IGFBPs secreted by a cell line.

For Better Understanding

Even though insulin — and more recently Long^{TM}R^{3}IGF-I — is used extensively for biopharmaceutical production, the cellular responses to either peptide in CHO cells are not well understood. To elucidate the mechanism for the increased potency of Long^{TM}R^{3}IGF-I compared with insulin, we examined the differences between their induced activation of the IR and the IGF-IR. In addition, we measured the subsequent phosphorylation of key antiapoptotic and mitogenic signaling molecules (Akt and MAPK) in CHO K1 (ATCC, www.atcc.org) cells and the dihydrofolate reductase (dhfr)–deficient cell lines CHO DG44 and DUKX-B11 (Institute of Medical and Veterinary Science, www.imvs.sa.gov.au).

Table 1: Homologous competitive binding assays were used to determine cell-surface receptor numbers (**9**). Receptor numbers were calculated using nonlinear regression and the equation of Cheng and Prusoff (**10**).

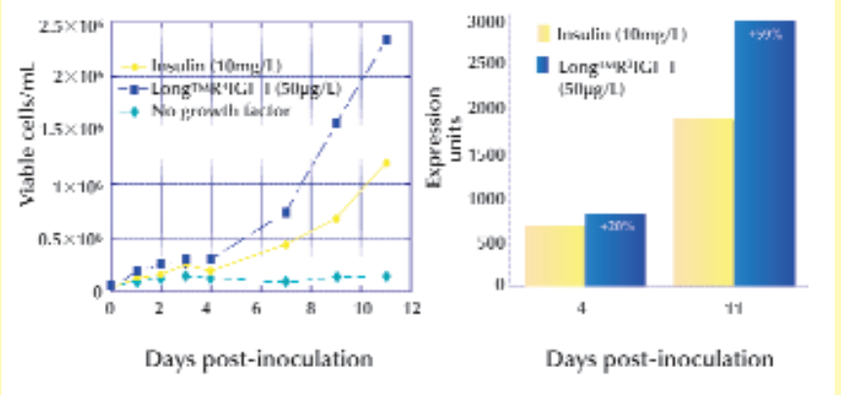
Insulin Receptor (IR) Numbers	
CHO K1	~3000/cell
DUKX-B11	~5000/cell
CHO DG44	undetectable
Type 1 Receptor (IGF-IR) Numbers	
CHO K1	~25,000/cell
DUKX-B11	~30,000/cell
CHO DG44	~40,000/cell

Quantifying Cell Surface Receptors: To compare the numbers of IGF-IRs and IRs on CHO cells, competitive binding assays using radiolabeled insulin and Long^{TM}R^{3}IGF-I were performed. All three cell lines showed similarly high levels of IGF-IRs on their cell surfaces (Table 1). CHO K1 and DUKX-B11 cell lines have 5- to 10-fold fewer cell surface IRs than IGF-IRs, as has been previously shown for the CHO K1 cell line (**21**). Cell surface IRs were undetectable on CHO DG44 cells by the current assay method. However, IR protein was detected in CHO DG44 cell lysate by a Western immunoblot (data not shown).

We also examined the influence of adaptation to protein-free medium on the levels of cell surface receptors in CHO K1 cells. Reducing serum from 10% to 2% had no effect on IGF-IR number. Preliminary experiments with CHO K1 cells adapted to growth in protein-free medium indicate a doubling in IGF-IR numbers. IR numbers were not determined for these cells.

Activation of Cell Surface Receptors: To elucidate the roles of the IRs and IGF-IRs in mediating insulin- or Long^{TM}R^{3}IGF-I–induced CHO K1 cell proliferation and survival, we examined the activation of each receptor after stimulation with various concentrations

Figure 1. Growth and production data for insulin versus Long^{TM}R^{3}IGF-I. CHO K1 cells producing a recombinant protein were adapted to growth in protein-free media. Cells were cultured in spinner flasks in SF medium (60 mL) containing either insulin, Long^{TM}R^{3}IGF-I, or no growth factor for a period of 11 days. Every 2–3 days, 80% of the conditioned medium was replaced with fresh medium.



of either growth factor. The concentrations of insulin and Long^{TM}R^{3}IGF-I used in these experiments were up to 10 times greater than required for cell culture. This is the standard approach taken for such experiments because of the insensitive nature of immunoblotting and the short time of stimulation. Therefore, the results from a short exposure to 500 µg/L of Long^{TM}R^{3}IGF-I can be extrapolated to a working concentration of 50 µg/L in cell culture. Likewise, stimulation with 100 mg/L of insulin can be extrapolated to 10 mg/L in cell culture.

Ligand binding studies with either receptor show transphosphorylation of the two ß subunits of the receptor, leading to receptor tyrosine kinase activation (**22**). We monitored the extent of receptor activation by examining the level of phosphorylation of the ß subunits of the IR and IGF-IR. Preliminary experiments revealed that phosphorylation of the receptors reached a maximum after 10 minutes of exposure to the growth factors and diminished thereafter. So this exposure time was used throughout the study.

Both Long^{TM}R^{3}IGF-I and insulin stimulated the phosphorylation of the IR and the IGF-IR (Figure 2). Long^{TM}R^{3}IGF-I was up to five times more potent than insulin at activating the IGF-IR, even at a 200-fold lower concentration. It also stimulated the IR at 200-fold lower concentrations than insulin. Given the relative affinities of IGF-I and insulin for the IR, this result was unexpected. Preliminary experiments demonstrated that the antibodies we used were specific for the respective phosphorylated forms of the IR or IGF-IR; therefore, cross-reactivity could not explain the result. However, the antibodies used for immunoprecipitation of the receptors may be recognizing IR/IGF-IR hybrid receptors. Cell surface IGF-IRs outnumber IRs by at least a factor of five. So a large proportion of the IRs may become phosphorylated due to the presence of such hybrids, which have a higher affinity for native IGF-I than for insulin (**23**).

Mediators and Suppressors of Apoptosis: IGF-IR and IR share extensive homology, so the

Figure 2. Phosphorylation of the IGF-IR or IR in CHO K1 cells after Long^{TM}R^{3}IGF-I or insulin stimulation. Lysates of stimulated cells were immunoprecipitated with the appropriate antireceptor antibody (anti-IGF-IRß or anti-IRß). Phosphorylation was detected by immunoblotting (IB) with antiphosphotyrosine antibody, and total protein by IB with anti-IGF-IRß or anti-IRß. Relative activation represent the ratio of phosphorylated protein to total protein.

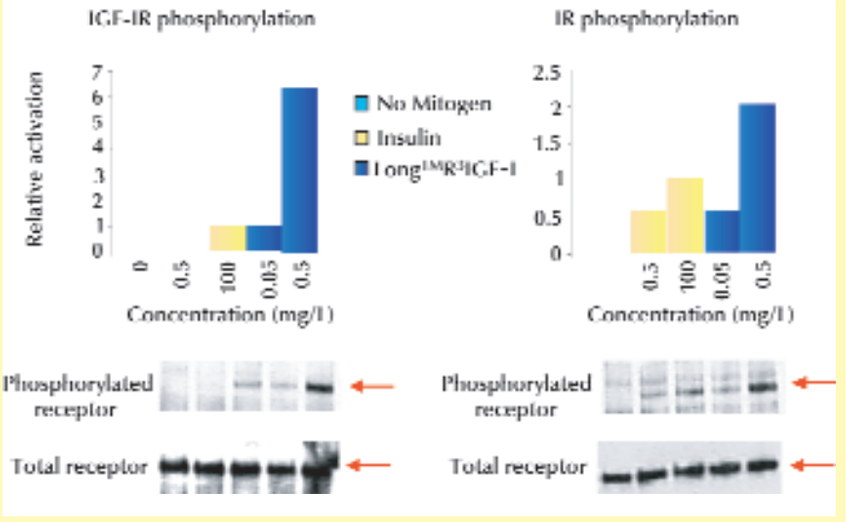


Figure 3. The intracellular signaling cascades activated by ligand-induced phosphorylation of either the IGF-IR or the IR. The two main pathways represented here are the Akt and MAPK cascades. It is important to note that only the IGF-IR is capable of mediating IRS-1 independent activation of the MAPK pathway, as denoted by the green arrow.

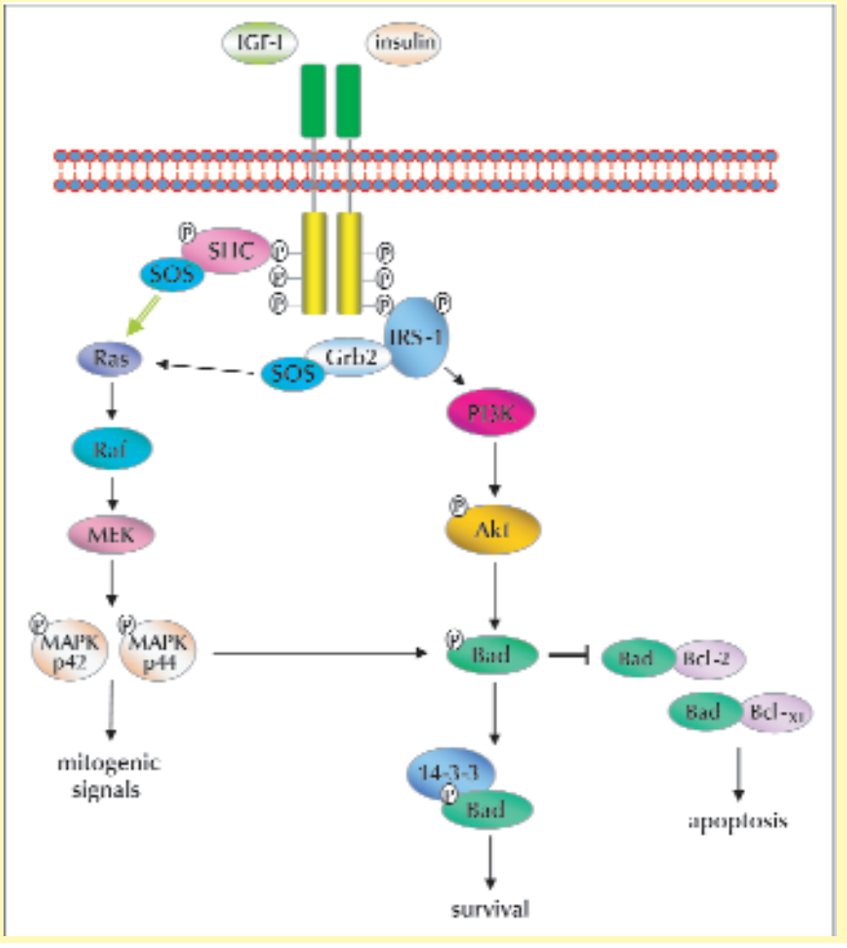
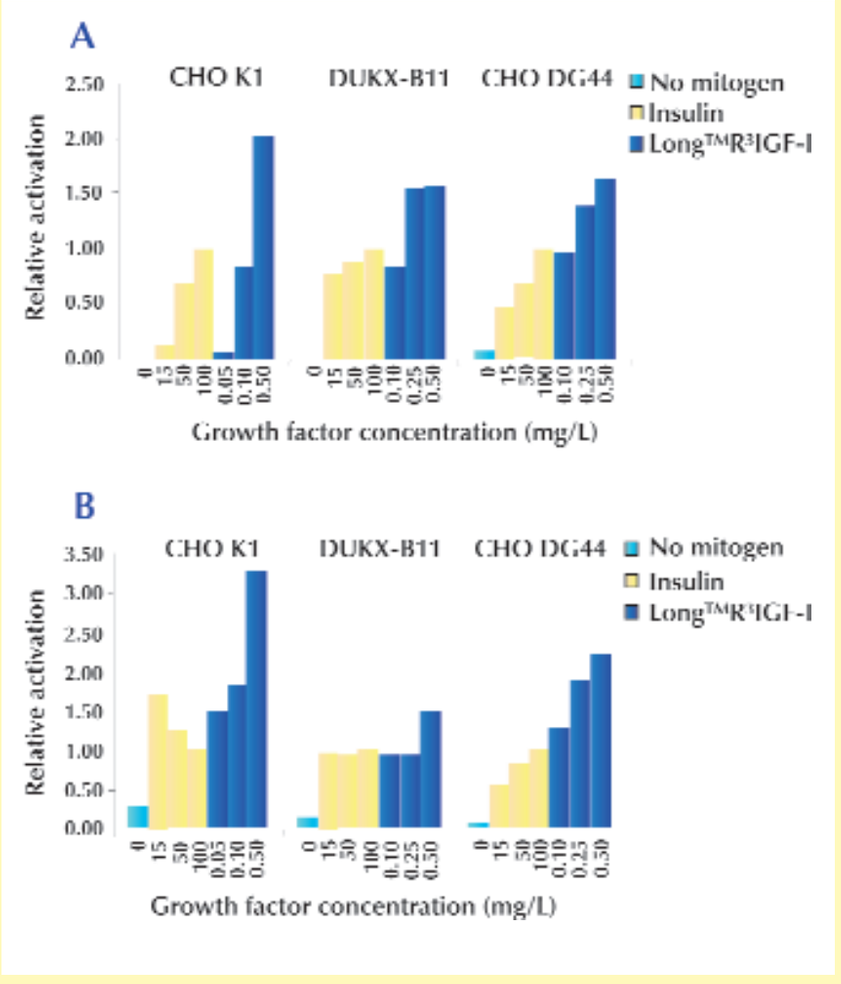


Figure 4. Phosphorylation of Akt (A) and p44/p42 MAPK (B) in CHO K1, DG44, and DUKX-B11 cells. Cells were grown in DMEM:F12, 10% FBS and starved of serum for 16 (DG44, DUKX-B11) or 24 (K1) hours before stimulation with various concentrations of insulin or Long^{TM}R^{3}IGF-I. Phosphorylated protein was detected by IB with either an anti-phospho-Akt antibody or an anti-phospho-p44/p42 MAPK antibody. Total protein was determined by IB with an anti-ß-actin antibody. Relative activation represent the ratio of phosphorylated protein to total protein (ß-actin). Data shown represents three independent experiments.



phosphorylation of either receptor can activate the same signaling pathways such as the Akt and MAPK cascades, thus stimulating phosphorylation of the same cellular substrates, including insulin receptor substrate (IRS)–1, IRS-2, and Shc, an adaptor protein in the MAPK cascade (**12**).

As illustrated in Figure 3, the serine/threonine kinase Akt/protein kinase B (a downstream effector of the PI3K signaling cascade) plays a central role in growth factor mediated cell survival and is reported to be the main signaling pathway through which IGF-IR exerts its antiapoptotic effect (**24**). In response to IGF-I, the IGF-IR interacts with IRS-1, which activates PI3K — thus leading to the phosphorylation and activation of Akt. In turn, Akt can promote cell survival through inhibitory phosphorylation of proapoptotic kinases, caspases, Bcl-2 family members, and transcription factors (**12**).

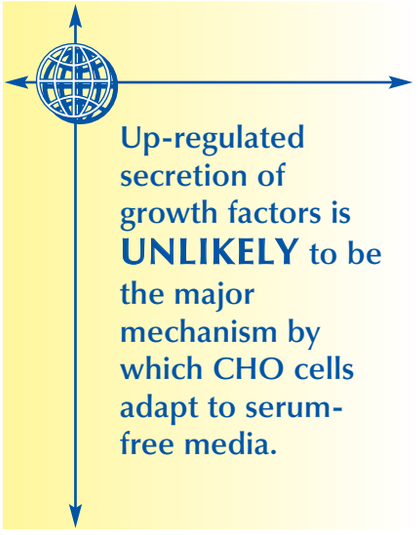
Specifically, the Bad protein (a proapoptotic member of the Bcl-2 family) is phosphorylated by the activated form of Akt at Serine 136 (**25**). The nonphosphorylated form of Bad is a heterodimeric partner for either Bcl-$_{XL}$ or Bcl-2, ablating their antiapoptotic effect and promoting cell death (**26**). However, once phosphorylated, Bad is bound by cytosolic proteins and can no longer interact with either antiapoptotic protein (**27**).

An alternative protective and mitogenic pathway is mediated by IRS-1 (Figure 3). Phosphorylated IRS-1 can be bound by the guanidine nucleotide exchange factor Grb2/SOS, initiating activation of the MAPK signal transduction pathway by sequential phosphorylation and activation of Ras, Raf, and MEK (**28**). However, whereas both insulin and IGF-I can induce MAPK phosphorylation through an IRS-1 mediated signal transduction pathway (**28**), insulin is not able to stimulate MAPK phosphorylation through the IR in the absence of IRS-1 (**29**).

IGF-IR can activate a third pathway to promote cell survival independent of IRS-1, giving it a specific signaling advantage over the IR (Figure 3). This pathway is initiated by the Shc adaptor proteins, another major substrate of the IGF-IR (**24**). Through SHC, IGF-I stimulation of the IGF-IR promotes activation of the ERK subfamily of MAPKs (p44/p42 MAPK), leading to either a proliferative response by upregulation of mitogenic proteins (**30**) or a cell survival response by phosphorylation of Bad (**31**).

Long^{TM}R^{3}IGF-I is a more potent stimulator of Akt and p42/p44 MAPK than insulin. We used the relative capacity of insulin and Long^{TM}R^{3}IGF-I to induce phosphorylation of either Akt or MAPK as a measure of the ability of each growth factor to promote CHO cell survival under SF conditions. Phosphorylation of Akt and p44/p42 MAPK in CHO K1, CHO DG44, and DUKX-B11 cells grown in 10% fetal bovine serum was assessed after stimulation with various concentrations of either Long^{TM}R^{3}IGF-I or insulin. These cells were serum starved for 16 (DG44/DUKX-B11) or 24 (K1) hours before growth factor addition. Long^{TM}R^{3}IGF-I stimulated both signaling intermediates to a greater degree than insulin at 200- to 1000-fold lower concentrations (Figure 4). This result was consistent across all three CHO cell lines tested.

We also assessed the ability of Long^{TM}R^{3}IGF-I and insulin to activate Akt and p44/p42 MAPK using CHO K1 cells adapted to growth in protein-free media. With these cells, the response to Long^{TM}R^{3}IGF-I and insulin was not as pronounced. Similar to cells grown in serum, Long^{TM}R^{3}IGF-I stimulated Akt to a greater degree than insulin at a 200-fold lower concentration (Figure 5).



However, MAPK phosphorylation was only slightly increased at all concentrations of both mitogens, relative to basal levels. It was difficult to compare potencies in this experiment because all doses produced similar responses.

Interestingly, the basal level of MAPK phosphorylation in SF-adapted CHO K1 cells was elevated compared to that for CHO cells maintained in serum-containing medium. In support of this observation, Reddy et al. (**32**) showed that basal levels of p44/p42 MAPK phosphorylation are elevated in cells adapted to growth in the absence of serum or any form of growth-factor stimulation.

Adaptation to SFM: Adaptation of CHO K1 to SF conditions does not result in significant changes in the number of cell surface IRs or IGF-IRs. Therefore, adaptation to growth without serum may rely on other mechanisms such as autocrine stimulation or changes in expression and/or phosphorylation of downstream signaling molecules. However, results in our own laboratory have demonstrated that serum-dependent CHO cells will not grow in conditioned media obtained from SF-adapted cell lines (unpublished data). Therefore, up-regulated secretion of growth factors is unlikely to be the major mechanism by which these cells have adapted.

Based on our findings, the observed increased basal level phosphorylation of MAPK could be one such mechanism used by CHO cells in adapting to SF conditions. Because Long^{TM}R^{3}IGF-I stimulated MAPK activation more readily than insulin in cells maintained in serum-containing medium, it should be better able to support adaptation of CHO cells to SF conditions. Indeed, we and others (**11**) have found that maintenance of cells in SFM can be facilitated by the addition of Long^{TM}R^{3}IGF-I without the need for the time-consuming and variable adaptation process of weaning cells from serum.

Stability in Cell Culture: Another advantage of Long^{TM}R^{3}IGF-I over insulin in cell culture is its stability. In the absence of any cells, both insulin and Long^{TM}R^{3}IGF-I are stable at 37 °C up to several days in culture. But we found that, in the presence of up to 1×10^6 CHO cells/mL in spinner flasks at 37 °C, insulin at 13 mg/L was almost completely degraded within four days. By contrast, the concentration of Long^{TM}R^{3}IGF-I with similar numbers of cells was reduced by only 20% after eight days in culture at 37 °C. It appears that, under batch or fed-batch culture conditions, insulin would need to be replenished more frequently than Long^{TM}R^{3}IGF-I.

Discussion

Efficient production of biopharmaceuticals requires maximum productivity from mammalian cell lines. Media formulations and feeding strategies for batch and fed-batch production cultures have led to reported yields as high as 5 g/L. The ability to maintain high cell viabilities under such conditions is paramount to achieving such productivity.

Insulin has been used in the SF manufacture of many biologics across a broad range of cell lines. Recently, Long^{TM}R^{3}IGF-I has shown the potential to improve the productivity of cell lines expressing biopharmaceutical proteins. Long^{TM}R^{3}IGF-I is a more potent growth factor than insulin in SFM. At concentrations 200-fold lower than insulin, Long^{TM}R^{3}IGF-I is better able to stimulate the IGF-IR and thus induce a higher level of activation of intracellular signaling molecules known to be responsible for promoting cell survival through inhibition of apoptosis.

Figure 5. Akt and MAPK phosphorylation in SF-adapted CHO K1 cells. Our methods of stimulation and detection are described in Figure 4. Relative activation represents the ratio of phosphorylated protein to total protein (ß-actin). Data shown represent three independent experiments.

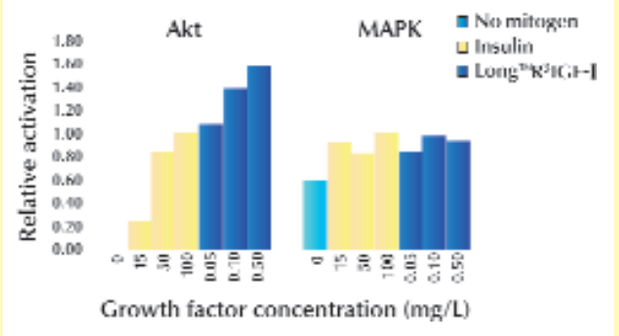
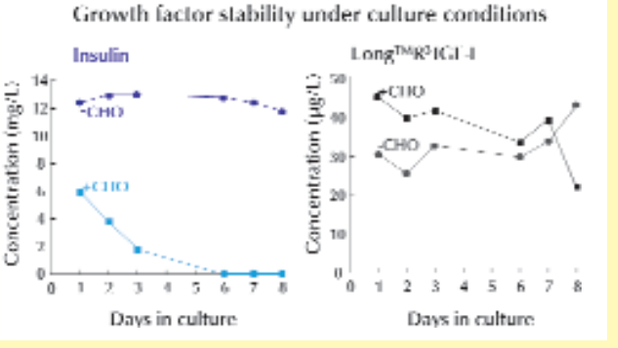


Figure 6. Spinner flask cultures, containing either insulin or Long^{TM}R^{3}IGF-I, inoculated with 1×10^5 CHO cells were incubated at 37 °C for eight days. Over that time, the viable cell number peaked at 1×10^6 and 1.4×10^6 cells/mL for cultures containing insulin or Long^{TM}R^{3}IGF-I, respectively. During the cultures, we determined the levels of each growth factor using commercial ELISA kits. Control spinner cultures containing no cells were also incubated and sampled similarly.



We have demonstrated that improved productivity can be gained as a consequence of the increased viability of cultures, a fact also demonstrated by other groups (**11**). These studies support the view that Long^{TM}R^{3}IGF-I is a more appropriate factor for biopharmaceutical production in CHO cells and that insulin, because of its low affinity for the IGF-IR, can achieve its potency only at much higher nonphysiological concentrations. This may also be the case in other cell lines — many of which are maintained with insulin at high concentrations (2–10 mg/L) — including hybridoma, Vero, HEK293, BHK, MDCK, and PER.C6 cells. Long^{TM}R^{3}IGF-I at much lower concentrations (10–100 µg/L) could replace insulin in those and many other cell lines to support the growth and survival of cells in SFM.

Long^{TM}R^{3}IGF-I is manufactured according to cGMP guidelines using a process that is in turn free of animal-derived components (from the master cell bank through to the final product). Long^{TM}R^{3}IGF-I is available exclusively through JRH Biosciences as a stand-alone ingredient or as a component of certain proprietary media for various cell types.

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