Quantitation of LONG®R³ IGF-I during production and purification of recombinant proteins from cell culture

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Abstract
LONG®R³ IGF-I is an analog of human insulin-like growth factor 1 that has been bioengineered for use in cell culture during production of recombinant proteins in human cells. LONG®R³ IGF-I comprises the 70 amino acids of IGF-1 with an arginine substitution for glutamic acid at amino acid 3 and a 13 amino acid extension peptide at the N-terminal end for a total of 83 amino acids. LONG®R³ IGF-I has a lower affinity for IGF binding proteins increasing its bioavailability to the IFG-1 receptor present on cells in culture. When supplemented in serum-free medium, it promotes cell proliferation and increases cell survival and productivity through better proliferative and anti-apoptotic signaling. A LONG®R³ IGF-I ELISA kit was recently developed to monitor the levels of LONG®R³ IGF-I in culture medium during the production process. The assay measures concentrations of LONG®R³ IGF-I in the 0.31 to 40 ng/ml range. The ready-to-use ELISA kit facilitates measurement of LONG®R³ IGF-I during optimization of the cell culture process and downstream processing purification.

The use of mammalian cell culture for the production of recombinant proteins has increased dramatically in recent decades, using serum-free and chemically defined cell culture media whenever possible during the production process. Bioactive proteins such as IGF and EGF, produced using recombinant DNA technology, have been widely used in today's production processes to replace animal-derived components such as FBS. This substitution has assisted efforts to minimize potential risk to product end-users, achieve greater consistency in process performance, and elevate process yields.

Use of chemically defined growth factors is essential for long-term growth and proliferation of cell lines in serum-free media formulations. These medium formulations have been used with numerous cell types including CHO, BHK, HEK 293, Vero, PER.C6, MDCK, and fibroblasts. Serum free medium has the advantage of lower batch-to-batch variability, reduced expense, and lower risk of viral contamination. It also simplifies downstream processing that removes serum proteins during purification of the recombinant product.

Insulin-Like Growth Factors

Serum-free medium formulations have included insulin for over 40 years (1, 2) due to its mitogenic properties. However, many formulations have substituted insulin-like growth factors (IGFs) for insulin, in large part because insulin's main role is the regulation of metabolic processes, whereas the IGFs function more effectively in promoting cell growth and differentiation (3). This difference makes the IGFs more suited for the purposes of expansion of cells in culture. The decision to use one member of the insulin/IGF signaling axis over the other is a complicated one, as the different molecules have overlapping roles.

The insulin-like growth factor (IGF) family includes insulin, IGF-1 and IGF-2. These structurally related peptides and their

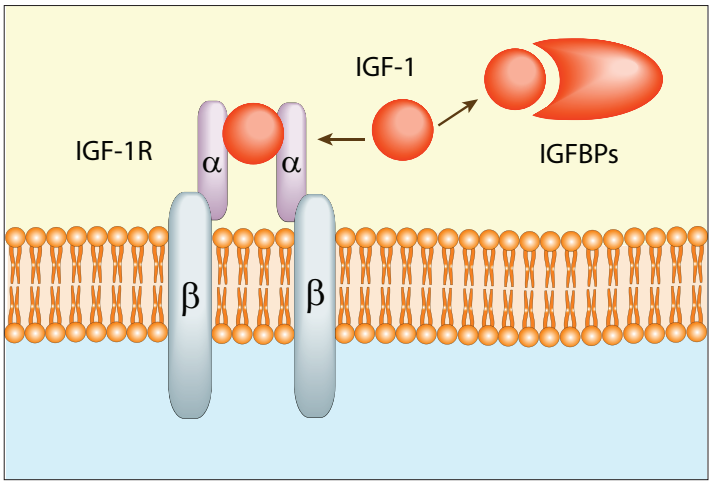

Fig. 1: *Binding of IGF-1 to its receptor IGF-1R and regulation by IGFBPs.*

receptors play an important and complex role in metabolic and growth functions in many organisms. The IGF family members are ligands for three receptors: the insulin receptor (IR), the IGF-1 receptor (IGF-1R), and the IGF-2 receptor (IGF-2R). The first two are structurally similar and are composed of dimers of disulfide-linked α and β chains (Fig.1), but the IGF-2R is structurally unrelated. Signals transduced through the IR result predominantly in metabolic processes, whereas those transmitted via the IGF-1R (Fig. 1) result primarily in growth and differentiation (4). IGF-2R has no known growth-promoting effects, and may act as a "sink" to control the levels and activity of IGF-2 *in vivo* (5). Furthermore, the different receptors have varying affinities for the three peptide growth factors. The IGF-1 receptor can bind insulin, thus potentiating insulin's mitogenic functions, although it binds insulin with much less affinity than IGF-1. Conversely, the insulin receptor can bind insulin with very high affinity but IGF-1 only weakly (3). The cross-binding activity of the receptors

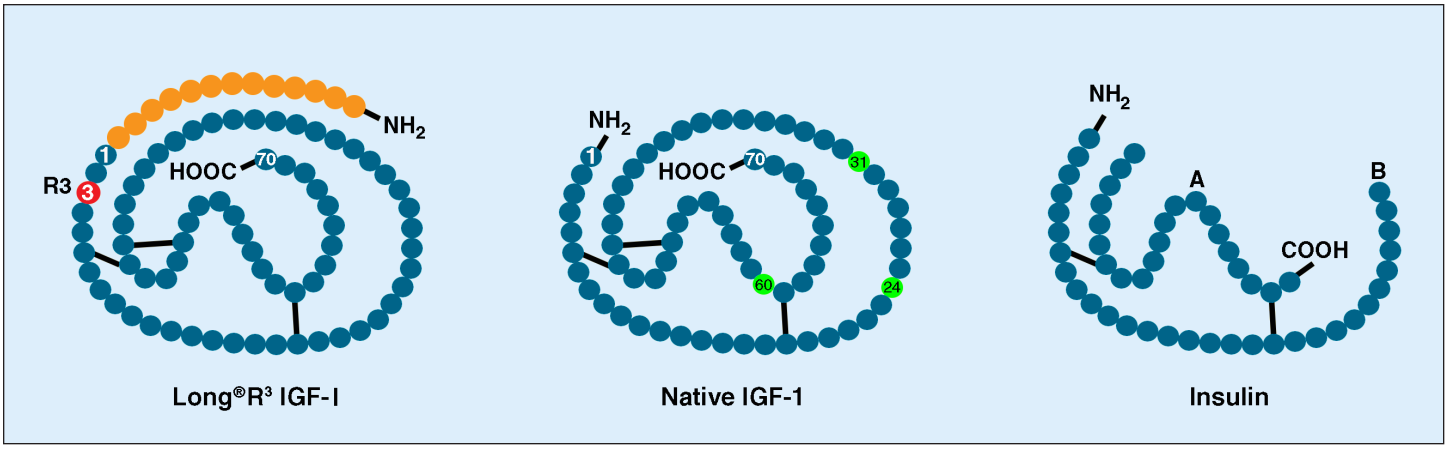

Fig. 2: *Comparison of the structure of LONG®R3 IGF-I, native IGF-1, and insulin. LONG®R3 IGF-I has an additional 13 aa peptide at the N-terminus (orange) and a Glu to Arg substitution at aa3 (red). The tyrosine locations at aa 24, 31, and 60 are shown on the native-IGF-1 molecule (green).*

for the various members of the IGF family leads to concentration dependent competition between the ligands. Indeed, it has been noted that much higher concentrations of insulin than IGF-1 are necessary to promote growth *in vitro*, possibly because the growth-control aspects of insulin are being potentiated through the IGF-1 receptor, which has a much lower affinity for insulin than the IR. This difference in the receptor's affinity for the two ligands argues for use of IGF-1 in lieu of insulin in serum-free medium formulations.

IGF-1 is a 70 amino acid peptide with 3 disulfide bonds (Fig. 2) (6). It has approximately 49% homology to insulin and 61% homology to IGF-2 (3). Approximately 80% of IGF-1 is produced in the liver and acts systemically (endocrine), whereas approximately 20% is produced locally in tissues and acts in a paracrine or autocrine fashion. The expression of the IGF-1 receptor occurs in numerous cell and tissue types (7). Thus, the IGF-1/IGF-1R pathway has wide-reaching effects in the organism and is implicated in anabolic processes from fetal growth, to muscle and bone repair, to various forms of cancer (4, 5). Signal transduction through the IGF-1R leads to several possible intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK) pathway, which generates a mitogenic signal, and PI3 Kinase-Akt pathway, which inhibits apoptosis and increases survival (5).

The strength and ubiquity of the IGF-1 signaling axis requires multiple levels of regulation, including receptor-ligand affinity, expression levels, and half-life of the peptides. Regarding half-life, IGF-1 is bound by several IGF binding proteins (IGFBPs), which play a regulatory role in its bioavailability (Fig. 1) (4, 8, 9). In general, the half-life of the IGF-1 peptide *in vivo* is limited to approximately 10 minutes (like insulin). However, IGF-1 is most often found in circulation in a complex with IGFBP-3 and the acid-labile subunit (ALS), which extends the half-life to up to 12 hours and acts as a reservoir for IGF-1. There are at least 6 known IGFBPs, with varying expression in different tissues, and variability between whether they are inhibitory or activating (4). *In vivo*, the IGFBP-3/ALS complex carries greater than 90% of the IGF in adult serum. The complex has a higher affinity for IGF-1 than does the IGF-1R, thus exerting an inhibitory effect on the IGF-1/IGF-1R interaction (8, 10). The seemingly opposite activity of the IGFBPs, both limiting IGF-1/IGF-1R interaction, as well as extending the availability of IGF-1 in circulation, is part of the complicated regulation of this powerful growth factor.

MFPAMPLSSL FVNGPRTLCG AELVDALQFV
CGDRGFYFNK PTGYGSSSRR APQTGIVDEC
CFRSCDLRRL EMYCAPLKPA KSA

Fig. 3: *Amino acid sequence of LONG®R³ IGF-I. Changes at the N-terminal end reduce binding to IGFBP and increase bioavailability to IGF-1R on cells. Substitutions for tyrosine (Y) at positions 24 and 60 interfered with binding to the IGF-1R and were not utilized in the design of the analog.*

LONG®R³ IGF-I

LONG®R³ IGF-I is an analog of IGF-1 that was developed for use in cell culture to promote cell growth and recombinant protein expression by cell lines. It maximizes the growth-promoting functions of IGF-1, but avoids the complicated interactions of the regulatory pathways that have evolved around it. The protein was created by substituting the negatively-charged glutamic acid (E) at amino acid 3 with a positively-charged arginine (R) and extending the N-terminal end of the peptide by 13 amino acids (MFPAMPLSSLFVN), a sequence derived from the Met-porcine growth hormone followed by the dipeptide Val-Asn (Fig. 2-3) (11, 12). These modifications reduced IGF-1 binding by IGFBPs and enhanced its biological activity. Development of the Long-Arg3 IGF-1 peptide was enabled by testing different analogs of IGF-1. Analogs with substitutions, deletions, or insertions at the N-terminal had greater biological activity than native IGF-1 (13-17). For example, Arg3 IGF-1, Gly3 IGF, and Des 1-3 IGF-1 (which has a deletion of the first 3 amino acids of IGF-1 [Gly-Pro-Glu]) also had greater growth promoting potential in comparison to native IGF-1, but not as great as Long-Arg3 IGF-1. In contrast, multiple substitutions at Tyr24, Tyr31, and Tyr60 (Leu24, Ala31, Leu60) had a great reduction in binding to IGF-1R (over 1000-fold) but only a slight effect on binding to IGFBPs (18, 19).

All cell types that have a growth response to insulin in cell culture have the potential to respond to LONG®R3 IGF-I, but at much lower concentrations. It has an increased half-life *in vitro*

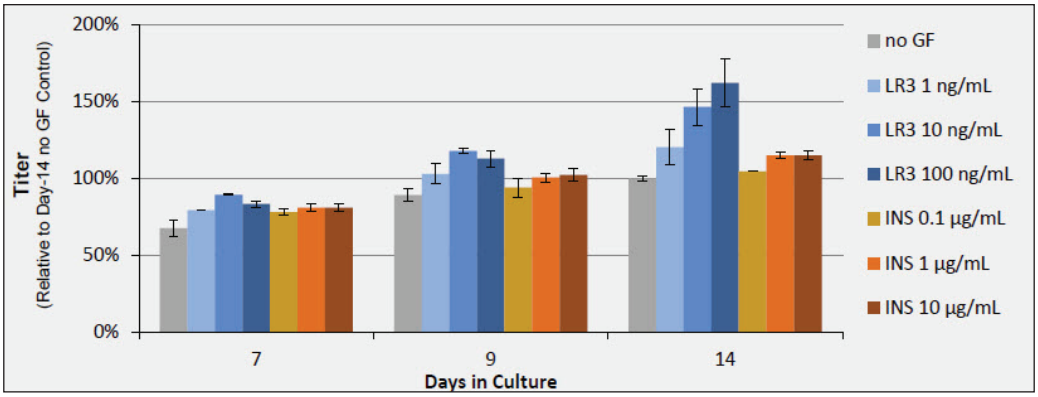

Fig. 4: *Graph of improved monoclonal antibody production by the addition of LONG®R³ IGF-I (LR3) vs. insulin (INS) or no growth factor (no GF) in a DP-12 CHO cell line. LONG®R³ IGF-I or insulin was added to custom serum-free CHO cell medium without any insulin or LONG®R³ IGF-I. DP-12 CHO cells supplemented with 100 ng/ml LONG®R³ IGF-I showed a 40% increase in titer vs 10 µg/ml insulin and a 62% increase relative to no growth factor. (Courtesy of Repligen Corporation)*

and *in vivo* and a low affinity for IGF binding proteins that make it ideal for both research and large-scale culturing (20-23). When supplemented in serum-free medium, it promotes cell proliferation and increases cell survival and productivity through better proliferative and anti-apoptotic signaling (Fig. 4).

LONG®R³ IGF-I ELISA

Quantitation of LONG®R³ IGF-I is important when developing cell culture processes, in order to optimize the concentration of LONG®R³ IGF-I used and feeding strategies. It is also important when developing a purification process, to demonstrate clearance of the growth factor from the supernatant. When LONG®R³ IGF-I was first developed the concentration was measured using an antibody-pair kit that included a LONG®R³ IGF-I standard (23-25). Recently, a complete ELISA kit for the detection of LONG®R³ IGF-I was developed to measure and optimize the concentration of the protein in cell culture, and to demonstrate clearance during protein purification processes in QC samples. The complete ELISA kit (Cell Sciences Cat # CKH194) includes a monoclonal antibody pre-coated microtiter plate, an *E. coli*-produced LONG®R³ IGF-I protein standard, a biotinylated detection antibody, reagent diluent, streptavidin-HRP, and TMB substrate. This kit format allows the assay to be run immediately, without additional plate coating and blocking steps (Fig. 5). The assay measures concentrations of protein in the 0.31 to 40 ng/ml range (Fig. 6). The capture and detection antibodies together are specific for LONG®R³ IGF-I but show an average of approximately 35% reactivity with the native IGF-1 sequence and virtually no reactivity with insulin (Fig. 7). Product information for the LONG®R³ IGF-I ELISA kit and recombinant protein for use in cell culture is listed in Table 1 on the following page.

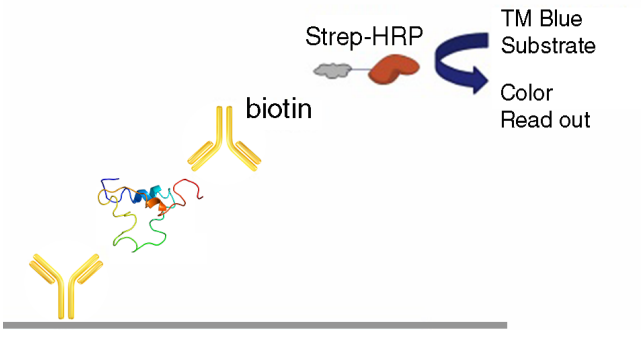

Fig. 5: *Schematic diagram of the LONG®R³ IGF-I Sandwich ELISA. The plate comes pre-coated with a monoclonal antibody to LONG®R³ IGF-I. Samples are detected with a biotinylated capture antibody and streptavidin-HRP. Development of the TMB substrate is stopped with sulfuric acid and the plate is read at 450 nm.*

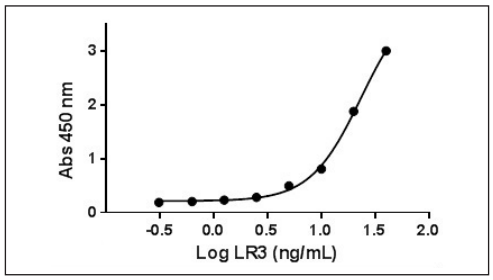

Fig. 6: *Representative LONG®R³ IGF-I standard curve. Data expressed using a 4-parameter curve. LONG®R³ IGF-I concentrations are plotted at 2 fold dilutions from 40 ng/ml (1.60 log units) down to 0.31 ng/ml (-0.51 log units). (Courtesy of Repligen Corporation)*

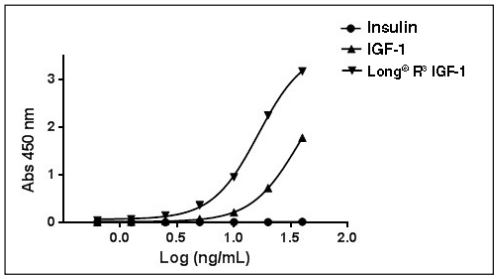

Fig. 7: *Comparison of growth factors as determined by the LONG®R³ IGF-I ELISA. IGF-I OD values measured at 10 to 40 ng/ml ranged from 20 to 56% of the LONG®R³ IGF-I OD values. The ELISA did not detect insulin at ng/ml concentrations. (Courtesy of Repligen Corporation)*

Table 1: LONG®R³ IGF-I ELISA and Related Products

Catalog No:	Description
CKH194	Human LONG®R³ IGF-I ELISA Kit
CRH060	LONG®R³ IGF-I (Recombinant Human) Lyophilized
CRH061	LONG®R³ IGF-I (Recombinant Human) Liquid
MAN100	Mouse Anti-Human LR3 IGF-1 Clone 6H5 mAb
MAO100	Mouse Anti-Human LR3 IGF-1 Clone 1A7 Biotin mAb

LONG® is a trademark of Repligen Corporation.

For more information about the latest IGF research reagents available from Cell Sciences, visit http://www.cellsciences.com.

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