Analysis of IgG heavy chain to light chain ratio with mutant Encephalomyocarditis virus internal ribosome entry site

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Immunoglobulin G (IgG) is a heterotetrameric protein assembled from two identical heavy chain (HC) and two identical light chain (LC) polypeptides. The HC and LC folding and assembly are a crucial step for IgG production. It is affected by the ratio of HC to LC expression (HC:LC). To date, the HC:LC ratio was analysed mainly by cotransfection of different amounts of two monocistronic HC and LC expression plasmids, an approach biased by different transfection efficiencies. To circumvent this problem, a series of Encephalomyocarditis virus internal ribosome entry site (EMCV IRES) variants with different translation efficiencies were created and used to mediate HC translation in bicistronic constructs. HC and LC were translated from the same mRNA, which provides a more accurate method for the evaluation of the optimal ratio of HC:LC. The results show that the IgG optimal expression levels were obtained when the IRES mediated translation efficiency of the HC was about 50% compared to the cap-dependent translation of the LC. A surprisingly sharp transition to low production was shown when the ratios were below 40%. This study provides a new method to investigate the production of heterodimeric proteins in mammalian cells and adds understanding to the mechanisms of IgG folding and assembly.

Keywords: antibody engineering/EMCV IRES/folding and assembly/IgG/Poly(C)

Introduction

During their production in mammalian cells, antibodies have to travel along the cellular secretory pathway to reach the final destination outside of the producing cells. The H and L chains first enter the endoplasmic reticulum (ER) as unfolded polypeptides, which are modified there and subsequently in the golgi apparatus (GA) to reach their final three-dimensional structure. Attributes such as conformation, structure of attached carbohydrates and oligomeric state not only control the functional properties but also are critical for intracellular transport. Newly synthesized polypeptides are retarded in exiting the ER towards the GA until they acquire a so-called export-competent conformation (Hurtley et al., 1989; Hammond and Helenius, 1995). Free antibody H chains are not exported (Morrison and Scharff, 1975), unless assembled with L chains into Ig molecules (Sonenshein et al., 1978; Leitzgen et al., 1997), while most L chains can be secreted as free monomer or dimer molecules (Shapiro et al., 1966; Skvortsov and Gurvich, 1968; Hannam-Harris and Smith, 1981; Hopper and Papagiannes, 1986; Dul et al., 1996). The HC:LC polypeptide ratio in the ER is a crucial factor influencing the rate of folding and assembly (Gonzalez et al., 2002). Most studies about the impact of heavy chain (HC) and light chain (LC) on Immunoglobulin G (IgG) folding and assembly in mammalian cells were done by cotransfection of two monocistronic HC and LC expression plasmids (Kaloff and Haas, 1995; Lee et al., 1999; Montano and Morrison, 2002; Schlatter et al., 2005). However, this approach is affected by varying transfection efficiencies. The discovery of internal ribosome entry sites (IRESs) provided a new tool for co-expressing multiple polypeptide chains of oligomeric or oligosubunit proteins in polycistronic expression systems.

The genome RNA of the cardiovirus and aphthovirus genera among the family Picornaviridae contains unusual, homopolymeric poly(C) tracts in the distal region of their 5’ untranslated regions (5’ UTRs). The poly(C) tract varies in length among different virus strain (Racaniello, 2001). Among cardioviruses, a longer poly(C) length is associated with higher virulence in animals (Duke et al., 1990; Martin et al., 2000) and recombinant mengoviruses with shortened poly(C) tracts have been used as attenuated live vaccines with proven genetic stability (Neal and Splitter, 1995; Osorio et al., 1996). In contrast to mengoviruses, Encephalomyocarditis virus (EMCV) with shortened poly(C) tracts were not attenuated, only the 20 to 30 bases of the 3’ terminal of the poly(C) were critical for growth (Hahn and Palmenberg, 1995; Martin et al., 1996). The commonly used EMCV IRES element has a poly(C) tract of about 20 bases. It was generally accepted that the poly(C) is functionally invisible to ribosome and does not contribute notably to virus viability (Duke et al., 1990; Rieder et al., 1993; Palmenberg and Osorio, 1994; Hahn and Palmenberg, 1995; Martin et al., 1996; Martin et al., 2000). We assumed that mutations in the poly(C) therefore might allow a very subtle regulation of IRES mediated mRNA translation.

To test the impact of intracellular HC expression on the IgG folding and assembly, we first made a mutational analysis of EMCV IRES including the poly(C) tract resulting in a series of IRES variants with different translation efficiencies. The IRES variants were then used to construct bicistronic IgG expression vectors. The LC expression levels were kept constant by positioning the L chain gene as the first cistron in the bicistronic construct, whereas HC expression levels...
were varied by using the EMCV IRES variants for cap-independent translation initiation, showing a very distinct dependence of production yields on the HC:LC ratio.

Materials and methods

Plasmids construction

All plasmids were constructed using standard *Escherichia coli* cloning methods as described (Sambrook and Russell, 2001). The sequences of EMCV-R IRES element refer to the literature (Duke et al., 1992). The construction of an IgG monocistronic expression plasmid pSH1, pSL1 and bicistronic plasmids pDLIY, pDYLH and pDHLH has been described previously (Li et al., 2007). The two bicistronic plasmids pDLIY and pDYLH encode an IgG LC and an YFP variant (Venus) as reporter gene with inverse cistron arrangement, respectively. The mutants of the EMCV-R IRES element were obtained by PCR amplification using synthetic oligonucleotides to induce a shortened or elongated poly(C) tract. Further IRES mutants were obtained by random error correction methods as described (Sambrook and Russell, 1989). The poly(C) sequence used in this study is a 30 C of the sequence. The sequence of the 20 bp oligonucleotide substitution for CCUCUCCCU(C)10 poly(C) tract is ACCCCAAAATAACCCAGAC. C0 indicates complete depletion of the poly(C) fragment. (C)n refers to modifications with the sequence CCUCUCCCU(C)n.

Cell culture and transfection

HEK 293T cells (ATCC No: CRL-11268) and CHO-K1 (ECACC No. 85051005) were grown as described previously (Li et al., 2007). Cells were seeded in 24-well or 6-well flat bottom tissue culture plates (Greiner bio-one, Frickenhausen, Germany) the day before transfection. The cells were transfected with a total amount of 1 µg plasmids DNA for each well of 24-well plate or 3 µg plasmids DNA for each well of 6-well plate using the Transfectin lipid (Bio-Rad, Hercules, USA) according to the supplier’s manual.

Flow cytometry analysis

Reporter gene YFP expression levels were analyzed using fluorescence activated cell sorters (FACS) analyzer (FACSCalibur; Becton Dickinson, San Jose, USA). The transfected cells were harvested after treatment with 5 mM ethylene-diaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2PO4, 2 mM KH2PO4, pH 7.2), washed with prechilled PBS containing 2% (v/v) FBS one time and resuspended in PBS containing 2% (v/v) FBS one time and resuspended in PBS containing 2% (v/v) FBS one time and resuspended in PBS containing 2% (v/v) FBS one time and resuspended in PBS containing 2% (v/v) FBS one time and resuspended in PBS containing 2% (v/v) FBS. Immunoblots were used to confirm IgG LC and HC secretion production, the transiently transfected HEK 293T cells were harvested and washed with prechilled PBS and centrifuged 300 x g for 5 min. Eight micro liter of culture supernatant was mixed with 2 µl 5 x SDS–PAGE Laemmli sample buffer (0.312 M Tris–HCl pH 6.8, 10% (w/v) SDS, 25% (v/v) β-mercaptoethanol, 0.05% (w/v) bromophenol blue) and denatured at 95°C for 10 min before being subjected to 12% (w/v) SDS–PAGE under reducing conditions. The separated protein samples were electroblotted onto PVDF membranes. These membranes were rinsed with PBS, blocked with 2% (w/v) skim milk powder in PBS (PBS-M) for half an hour, stained with goat anti-human kappa LC specific alkaline phosphatase (AP) conjugated antibody (1:1000 in PBS-M)

Immunoblot

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Point mutations were found in the region from the F domain to the K domain of EMCV IRES. Their effect on YFP translation efficiency ranged from 5 to 110% activity compared to wild type IRES mediated translation. Mutation A345G and the combination of A345G with A525G had only a moderate effect to IRES mediated translation efficiency, supporting a previous report, which showed that the 5' region of EMCV IRES upstream of the intact H domain and the lower part of stem I are not required for translational functionality, if the loop (base 527 to 591) on the top of stem I was not disrupted (Duke et al., 1992). The combination of mutations A345G, U394C and G756A and the combination of A499G and U778C significantly decreased translation efficiency to 5 and 10%, respectively, compared to wild type IRES.

To compare the translation efficiency of the IRES variants to that of cap-dependent mRNA translation, the IRES mediated YFP expression levels were compared with cap-dependent YFP expression levels from a bicistronic construct with inverse cistron arrangement (Fig. 2A). The relative expression.

Results

The impact of mutations on IRES mediated mRNA translation

To obtain a panel of IRES variants with a subtle difference in translation efficiency for the study of the impact of H chain on mammalian IgG production, we made a mutational analysis of EMCV IRES. The resulting IRES variants were arranged to mediate a bicistronic expression with a LC gene as the first cistron and YFP variant (Venus) reporter gene as second cistron (Fig. 1). YFP expression efficiencies were evaluated in live HEK 293T cells by FACS 48 h after transfection. The modification of the poly(C) region between base 270 and 280 only slightly effected IRES mediated YFP translation. The deletion of the 20-base poly(C) tract reduced YFP expression to 30%. The 20-base nucleotide fragment from the upstream of 5' EMCV poly(C) tract was used to replace the 20-base poly(C); this leads to further reduction of IRES mediated YFP expression to 15% compared to the IRES element with the 20-base pairs of the wild type poly(C) tract. In contrast, when the 20-base pair oligonucleotide fragment was added 5' of base 260 of the EMCV IRES, no apparent effect was found on the reporter gene expression.

and/or goat anti-human IgG gamma chain specific AP conjugated antibody (1:1000 dilution in PBS-M) (Sigma, Steinheim, Germany) at R.T. for 1–2 h and developed in BCIP/NBT (Roth, Karlsruhe, Germany) substrate solution. All washing was done 3×5 min with PBS. The blots were analysed with Gel Doc 2000 image analysis system and quantified with a Quantitative one software (Bio-Rad, Hercules, USA) according to a standard IgG with known concentration.
translation efficiency of the IRES variants ranged from 0.03 to 0.6 compared to cap-dependent translation.

The secretion levels of the cap-dependent translated LC, encoded by the cistron upstream of the IRES were analyzed by immunoblot for control. No significant differences were observed between the different constructs (Fig. 2B).

**The impact of the HC on IgG production in transiently transfected HEK 293T cells**

To investigate the optimal ratio of intracellular HC and LC expression levels for IgG production, the second cistron YFP reporter gene was replaced by a HC gene. Thereby, the LC expression levels were expected to be kept constant by positioning the LC gene as the first cistron in the bicistronic constructs, whereas the HC expression levels should depend on the particular EMCV IRES mutant used. HEK 293T cells were transiently transfected with these bicistronic IgG expression plasmids. Antibody expression levels in the supernatant were evaluated by ELISA. The constructs containing an IRES variant with a relative translation efficiency (when compared to cap-dependent translation) lower than 0.2, IgG production was very inefficient, yielding only trace amounts of secreted IgG (Fig. 3). However, when the relative translation efficiency of the HC was above 0.4, the IgG production levels increased rapidly. The maximal IgG yield was obtained from a construct containing an IRES variant with a relative translation efficiency of 0.5. IgG production was not improved further in transiently transfected HEK 293T cells when HC translation was further increased either in a construct with a more efficient IRES element (Fig. 3) or the construct pCMVL-EF1αH with a combination of two separate promoters for HC and LC expression (Fig. 6A and 6B). The EF1-α promoter has been described to be at least two times more efficient than the CMV promoter (Li et al., 2007).

To compare the intracellular levels of the HC and LC, the cell lysate of transiently transfected HEK 293T cells were analyzed by ELISA. The signal from the IRES mediated intracellular HC was reduced gradually with the use of lower efficient IRES variants (Fig. 4). When the relative translation efficiency ratio of HC to LC reached 1:2, the intracellular retention molar ratio of HC to LC was about 1. This corresponded to the optimal IgG yield in the supernatant.

**The impact of HC on IgG production in transiently transfected CHO-K1 cells**

CHO cells are the most widely used cell lines for biopharmaceutical production. For recombinant protein production, host cells may exert different impact on the efficiency of target protein folding, assembly and production. In order to facilitate IgG production in CHO cells, based on the analysis above, we analysed four different EMCV IRES variants Iw, I6, I8 and I12 in transiently transfected CHO-K1 cells. The IRES variants mediated YFP gene expression showed no apparent difference, the relative translation efficiencies ranged from 0.31 to 0.33 (Fig. 5). Compared to transiently transfected HEK 293T cells, the EMCV IRES variants were less efficient for second cistron gene expression in CHO cells. To analyse the impact of HC on IgG production in CHO cells, a series of bicistronic plasmids with different cistron arrangement and the combination of two promoters in

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**Fig. 3.** Effect of the relative translation efficiency of HC:LC on IgG production in HEK 293T cells. IgG was transiently expressed from bicistronic vectors, where the LC was positioned as first cistron and translated under a cap-dependent mechanism; the HC was in the second cistron and translated under IRES-driven internal initiation mechanism. The IgG concentration in the supernatant was determined by ELISA. The IRES mediated relative translation efficiencies refer to Fig. 2A.

**Fig. 4.** Intracellular HC and LC levels in CHO cells as determined by ELISA. About 10⁶ of transiently transfected CHO-K1 cells were lysed in 200 μl lysis buffer 48 h after transfection, the concentration of HC and LC in the cell lysate was determined by ELISA.

**Fig. 5.** YFP expression in CHO cells from bicistronic constructs with different IRES variants. YFP fluorescence intensity was determined by flow cytometry, data were acquired using a cytomics FC 500 with CXP software and analysed with WinMDI. Error bars represent standard deviation of three independent experiments.
The yields of functional secretory IgG strongly depend on the proper folding and assembly in the ER, relying on chaperone systems that monitor and assist in the folding process. HC binding protein (BiP), protein disulfide isomerase (PDI) and the ratio of HC and LC polypeptide intracellular expression levels in the ER are believed to be crucial factors which influence the rate of folding and assembly (Gonzalez et al., 2002). In this study, we created a series of EMCV IRES variants with different translation efficiencies, which were used to study the impact of relative the ratio of HC to LC on IgG production. A fluorescent reporter (YFP) gene was used as second cistron to evaluate the EMCV IRES variants mediated translation efficiency, with LC as first cistron.

A number of studies have shown the importance of both RNA secondary and tertiary structures in viral cap-independent translation (Jang and Wimmer, 1990; Le et al., 1993; Haller and Seamler, 1995; Wang et al., 1995; Hoffman and Palmenberg, 1996; Honda et al., 1996; Witwer et al., 2001). We found that modification of the 20 bases at the 3' part of the poly(C) tract of the EMCV IRES element can affect IRES mediated protein translation efficiency in transiently transfected 293T cells. Our study suggests that this 20 bp fragment of the poly(C) tract plays an important role in the IRES mediated translation efficiency as it could not be functionally replaced by a different 20 bp oligonucleotide sequence. It indicates that the EMCV IRES mediated translation efficiency is likely to be affected by the sequence of the cistron upstream of the IRES if the poly(C) tract was completely deleted. The impact of mutations inside the IRES element was consistent with previous reports. Those mutations found in the conserved J, K domain reduced the IRES mediated translation efficiency to 5–10% compared to the wild type IRES element, whereas mutations in other stems or loops had only minor effects (Duke et al., 1992; Hoffman et al., 1995).

The mutational analysis of EMCV IRES allowed to prepare a series of variants with relative translation efficiencies ranging from 0.03 to 0.6 compared to cap-dependent translation efficiency. As the EMCV IRES mediated cap-independent translation efficiency is lower than cap-dependent translation, it was expected that the IgG bicistronic expression would depend on the H chain expression levels in bicistronic expression vectors with LC as first cistron and HC as second cistron. Promoter strength or local effects were avoided as the L chain and H chain are translated from the same mRNA. It was found that an optimal IgG transient production from HEK 293T cells was obtained when an IRES variant with a relative translation efficiency of 50% compared to cap-dependent translation was used. Therefore, it can be assumed that the optimal IgG yield was obtained when HC and LC translation efficiency was in the range of 1:2 (Fig. 3). Significantly, in HEK 293T cells, the IgG production was not increased when a more efficient IRES variant or, in a monocistronic setup, the stronger EF1-α promoter was used to drive HC expression while LC was kept driven by CMV promoter in one plasmid. The EF1-α promoter was shown to be at least two times more...
efficient than the CMV promoter for IgG mammalian expression (Li et al., 2007).

Analysis of intracellular HC and LC levels in this study showed that an intracellular HC:LC molar ratio about 1 corresponded to an optimal IgG yield in the supernatant. In transiently transfected CHO-K1 cells, EMCV IRES was less efficient than in HEK 293T cells. The relative translation efficiency of the most efficient IRES variant was about 0.33, the IgG production from CHO cells was increased significantly by using the monocistronic pCMVL-EF1aH construct. Here, the corresponding intracellular HC:LC molar ratio was about 1.7.

It was reported that an excess of intracellular LC polypeptide resulted in improved Mab production (Bibila and Flickinger, 1991; Whiteley et al., 1997; Schlatter et al., 2005). Interestingly, in our study, the bicistronic IgG expression constructs with HC as first cistron upstream of the IRES element, which leads to an excess of HC expression (with an intracellular HC:LC ratio about 8) gave a more efficient IgG production in transiently transfected HEK 293T cells than the constructs with a less efficient IRES variant (pDLI4H, pDLI9H, pDLI10H and pDLI15H, with relative translation ratios of HC:LC of around 0.1). In previous publications, it was shown that a HC:LC ratio higher than 1 can generate an infinite assembly time (Gonzalez et al., 2002), and that an excess of LC over HC polypeptide in the ER facilitates IgG production by minimizing accumulation of unfolded HC polypeptide, thus increasing the rate of IgG folding and assembly and reducing stress in the ER (Bertolotti et al., 2000; Schlatter et al., 2005). In this study, however, we observed a significant IgG production in HEK293T cells when intracellular HC was in excess over LC. However, in CHO cells, intracellular excess of HC led to decreased yields of IgG in the supernatant, which illustrates that different mammalian cell lines may show quite different behavior with regard to the effect of HC:LC ratios on antibody productivity.

Acknowledgements

We would like to thank Jürgen Bode, Junhua Qiao from German Research Centre for Biotechnology, and Nina Strebe from Technical University of Brunswick for their kind help on FACS scanning during this work. We would like to thank Atsushi Miyawaki from the Laboratory for Cell Function and Dynamics, Brain Science Institute, Japan, for his generous gift of the YFP gene Venus. CZ was supported by the International Graduate College. We would like to thank Juergen Bode, Junhua Qiao from German Research Centre for Biotechnology, and Nina Strebe from Technical University of Brunswick for their kind help on FACS scanning during this work. We would like to thank Atsushi Miyawaki from the Laboratory for Cell Function and Dynamics, Brain Science Institute, Japan, for his generous gift of the YFP gene Venus. CZ was supported by the International Graduate College.