SECRETION OF BIOLOGICALLY ACTIVE RECOMBINANT HUMAN ERYTHROPOIETIN IN MAMMALIAN CELL CULTURE

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Erythropoietin (EPO) is the main hormone involved in the process of erythroid differentiation. Human EPO is a highly glycosilated protein, and its natural source for purification is the urine of patients with severe aplastic anemia. The need of hEPO in the world is growing dramatically, while production from urine is step-limiting. The protein is too complex to be produced in active form by recombinant technology in bacteria or yeast. The only reliable source for recombinant hEPO are mammalian cells, where the cloned hEPO cDNA had been expressed (1, 2). Here we report on the secretion of biologically active rhEPO by Chinese hamster ovary (CHO) cells.

METHODS

The full lengh cDNA of hEPO was cloned from fetal kidneys and placed in the expression plasmid pAD30 (3). This construct included ori, enhancer and polyA sequences from SV40, adenovirus type II major late promoter, tripartite leader and VA genes, and a selection gene coding for the murine enzyme dehydrofolate reductase. CHO dhfr cells were transfected by calcium phospate precipitation using standard procedures. Cells were cultured in MEM alpha (Gibco), supplemented with 2 mM glutamine, antibiotics and 10% v/v of fetal calf serum (Gibco).

Selection of dhfr resistant clones, was carried out in MEM without nucleosides (NO^-). Amplification of the gene was accomplished by increasing the proportion of metrotrexate (MTX) in the medium from 20 nM to 6.4 μM. Supernatant from the cell cultures were collected after 48 h of culture in absence of serum. The rhEPO was partially purified by affinity chromatography, on Blue Sepharose (Pharmacia, Sweden) and was detected by either a commercial ELISA test (Boehringer Manhein, FRG) or in immunodots with a rabbit heteroserum against a peptide of hEPO (Sigma), conjugated with BSA as carrier. To analyze the biological activity of the secreted rhEPO, the method of Krystal (4) was used for *in vitro* experiments, while the *in vivo* activity was assayed by the polycistemic-mice test. Northen, Southern and Western blots were performed as routine procedures (5).

RESULTS AND DISCUSSION

Recombinant hEPO was detected after transient transfection of CHO cells, this indicated that the plasmid was actively transcribed in the cell system. A stably transformed line of CHO dhfr, was established by selection for the dhfr gene product. Of the colonies obtained, 45 were able to grow in MEM NO^- (transfection efficiency of 6.4×10^{-5}). The transfected gene was amplified by 4.8-fold in MTX, the copy number of the amplicon, and the productivity after amplification are shown in the table.

Purification of rhEPO yielded a reproducible, more than 95% purity product. The identity of the purified EPO was confirmed by Western blotting. Partial (~33%) sequencing of the N-terminus had been done, and the sequenced aminoacids of the protein matched exactly those of the natural protein.

Studies are on the way to fully sequence the protein, and to characterize the structure of the oligosaccharide chains by nuclear magnetic resonance. In conclusion, we acchieved the secretion of biologically active recombinant human EPO in CHO cells.

Concentration of MTX	Copy number (2n genome)	Conc. of EPO (μg/mL)	Productivity (mU/cell/day)	Specific biological activity (Umg^{-1})	
				in vitro	*in vivo*
nill	24	30	5.4	yes	yes
6.4 μM	117	100	18,0	10^5	8×10^4

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EXPRESSION AND STRUCTURAL ANALYSIS OF 14-3-3 PROTEINS

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INTRODUCTION

The 14-3-3 family of proteins was so named due to its migration on position on two-dimensional DEAE and gel electrophoresis (1). These proteins all have a molecular mass of around 30 kDa and exist as dimmers (2). To date, seven to eight mammalian brain isoforms of 14-3-3 have been described, named alpha-eta after their respective elution positions on HPLC (3). Five of these have been sequenced (4) and the alpha and delta isoforms are identical in primary structure to the beta and zeta isoforms respectively, but differ only in a post-translational modifications (5). The 14-3-3 family is highly conserved and individual isoforms differ by 1 to 5 mainly conservative amino acid substitutions. Isoforms have also been described from other mammalian tissues which are absent or present at low levels in the brain. These include an isoform found in T-cells (6) and one found in epithelial cells (7, 8). These have been named tau and epsilon respectively (5).

In this multi-disciplinary study we have used isoform-specific antibodies to analyse the domain structure of members of the 14-3-3 family after digestion with proteases. We concentrated on two isoforms of 14-3-3: tau, which is found at low levels in all tissues tested to date, and epsilon, which is found at high levels in brain and other tissues. Intact tau isoform and various deleted forms of tau were expressed in *E. coli*. Regions of the protein involved in dimerisation and membrane attachment were determined, and the nature of the phosphorilation by protein kinase C was analysed. In this way we have started to dissect the structure of 14-3-3 proteins and their function as regulators of protein kinase C.

RESULTS AND DISCUSSION

Using antisera specific for the N-termini of 14-3-3 isoforms described previously and an additional antiserum specific for the C-terminus of epsilon isoform, protease digestion of intact 14-3-3 showed that the N-terminal half of 14-3-3 (a 16 kDa fragment) was an intact, dimeric domain of the protein. This was confirmed by electrospray mass spectrometry.

Two isoforms of 14-3-3, tau and epsilon, were expressed in *E. coli* and secondary structure was shown by circular dichroism to be identical to wild-type protein. Expression of N-terminally-deleted epsilon 14-3-3 protein showed that the N-terminal 26 amino acids are important for dimerisation. Intact 14-3-3 is a potent inhibitor of protein kinase C, but the N-terminal domain does not inhibit PKC activity. Site-specific mutagenesis of several regions in the N-terminal of the tau isoform of 14-3-3 did not alter its inhibitory activity. 14-3-3 proteins are found at high concentration on synaptic plasma membranes. This binding is mediated through the N-terminal 12 kDa of 14-3-3. Intact 14-3-3 are phosphorilated by protein kinase C with a low stoichiometry, but truncated isoforms are phosphorilated much more efficiently by this kinase. This may imply that the proteins may adopt a different structural conformation, possibly upon binding to the membrane, which could modulate their activity.

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