Research Article

Cellular repressor of E1A-stimulated genes is a *bona fide* lysosomal protein which undergoes proteolytic maturation during its biosynthesis

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ARTICLE INFORMATION

ABSTRACT

Cellular repressor of E1A-stimulated genes (CREG) has been reported to be a secretory glycoprotein implicated in cellular growth and differentiation. We now show that CREG is predominantly localized within intracellular compartments. Intracellular CREG was found to lack an N-terminal peptide present in the secreted form of the protein. In contrast to normal cells, CREG is largely secreted by fibroblasts missing both mannose 6-phosphate receptors. This is not observed in cells lacking only one of them. Mass spectrometric analysis of recombinant CREG revealed that the protein contains phosphorylated oligosaccharides at either of its two *N*-glycosylation sites. Cellular CREG was found to cosediment with lysosomal markers upon subcellular fractionation by density-gradient centrifugation. In fibroblasts expressing a CREG-GFP fusion construct, the heterologous protein was detected in compartments containing lysosomal proteins. Immunolocalization of endogenous CREG confirmed that intracellular CREG is localized in lysosomes. Proteolytic processing of intracellular CREG involves the action of lysosomal cysteine proteinases. These results establish that CREG is a lysosomal protein that undergoes proteolytic maturation in the course of its biosynthesis, carries the mannose 6-phosphate recognition marker and depends on the interaction with mannose 6-phosphate receptors for efficient delivery to lysosomes.

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Introduction

Cellular repressor of E1A-stimulated genes (CREG) is a glycoprotein proposed to participate in a wide range of cellular processes. Among other cellular activities, CREG has been found to antagonize transcriptional activation and cellular transformation by the adenoviral E1A oncoprotein [1], to induce differentiation while attenuating cellular proliferation [2], to regulate the levels of the signalling kinases ERK 1/2 [3], and to mediate the glucocorticoid-induced proliferation of ileal epithelial cells [4]. These observations have led to the notion that CREG may play an important role in the control of cell growth and differentiation. However, the biochemical basis for its cellular activities has not been unravelled so far.

In a search for potential interaction partners, CREG was found to bind directly to the mannose 6-phosphate/insulin-like growth factor II receptor (M6P/IGF2R). Since this interaction was shown to depend on N-glycosylation of CREG, it was inferred that CREG binds to M6P/IGF2R via M6P residues located in its N-glycans [5]. Other studies have shown that CREG interacts with M6P/IGF2R in an M6P-dependent manner [6–9]. Recent results indicate that human CREG carries M6P moieties at two of its three N-glycosylation sites [10]. By analysing CREG activity in cells lacking M6P/IGF2R, evidence was provided that this receptor is involved in CREG-induced growth inhibition of mammalian cells [5]. However, it has been put forward recently that CREG could interact preferentially with the 46-kDa mannose 6-phosphate receptor (MPR46), the second M6P receptor present in mammalian cells [11].

The three-dimensional crystal structure of recombinant human CREG has been recently elucidated, revealing structural homology to a family of flavin mononucleotide (FMN)-binding proteins [12]. However, the putative FMN-binding pocket of the protein is sterically blocked and thus not capable of interacting with this nucleotide. Nevertheless, this region of CREG could represent a ligand-binding site since removal of segments of this domain by site-directed mutagenesis abolished the growth-suppressive properties of the protein [12]. CREG forms a homodimeric complex, with the three potential N-glycosylation sites mapping to a confined patch on the surface of the protein. If modified with M6P residues, these clustered N-glycans could well account for a tight interaction between CREG and M6P receptors [12].

The bulk of glycoproteins carrying M6P residues in their carbohydrate moieties are lysosomal enzymes and other proteins resident within lysosomes. Intracellular trafficking of M6P-modified proteins to lysosomes depends mostly on the M6P receptor (MPR) system, since cells with either impaired synthesis of the M6P recognition marker or a complete MPR deficiency fail to retain most of their newly synthesized lysosomal proteins [13–15]. Studies on MPR46- and/or M6P/IGF2R-negative cells have indicated that both receptors are necessary for efficient lysosomal targeting. It has been suggested that the two receptors complement each other by binding to distinct subpopulations of soluble lysosomal proteins [16]. However, sorting via M6P/IGF2R is generally far more efficient than by MPR46, demonstrating that the former is the main lysosomal targeting receptor in mammalian cells.

In this study, we have investigated the subcellular localization of CREG and its biosynthetic transport in normal and MPR-deficient murine fibroblasts. Furthermore, we have directly assessed whether CREG carries M6P residues within its N-glycans. In contrast to previous studies suggesting that CREG is a secretory protein, our results establish that CREG resides in lysosomes and undergoes proteolytic maturation due to the action of lysosomal proteinases. Furthermore, we show that murine CREG contains phosphorylated oligosaccharides at both N-glycosylation sites, and that both M6P receptors are capable of mediating the intracellular retention of CREG in mammalian cells.

Materials and methods

Reagents

Oligonucleotide primers were synthesized by VBC Biotech Services (Vienna, Austria). Restriction enzymes were purchased from New England BioLabs (Beverly, MA) and Fermentas (St. Leon-Rot, Germany). UDP-[^{3}H]galactose (5–20 Ci/mmol) and Percoll were provided by GE Healthcare (Little Chalfont, U.K.). Endo H and PNGase F were from Roche Diagnostics (Mannheim, Germany). Prestained molecular mass standards and enhanced chemiluminescence western blotting reagents were obtained from Bio-Rad (Richmond, CA). Bovine serum albumin (fraction V), chicken egg ovalbumin (grade V), E-64, leupeptin, mannose 6-phosphate, 4-methyl-umbelliferyl-β-N-acetylglucosaminide, pepstatin A, PMSF, and sodium β-glycerophosphate were purchased from Sigma (St. Louis, MO). The cysteine proteinase inhibitors CA-074 and Z-LVG-CHN$_2$ were kindly provided by Dr. Nobuhiko Katunuma (Tokushima Bunri University, Japan) and Dr. Magnus Abrahamson (University of Lund, Sweden), respectively. All other chemicals were of reagent grade.

Antibodies

The CREG-derived peptides CREG30 (CS[30]GRGGRDHGDWDVDRRLPPL[49]) and CREG92 (CS[92]DGPPGEGTGEPYMYLSPLQQAVSDLQEN[120]) were synthesized in-house, purified and then conjugated to keyhole limpet hemocyanin as previously described [17]. The peptide conjugates as well as purified recombinant murine CREG produced in insect cells (see below) were then used to immunize rabbits (done by Gramsch Laboratories, Schwabenhausen, Germany). Antibodies were purified by affinity chromatography with immobilized peptides or recombinant CREG, respectively, essentially as reported [17,18], using either 0.1 M glycine/HCl buffer (pH 3.5) or 3 M KSCN in 0.5 M NH$_4$Cl/NH$_3$ buffer (pH 10.0) for elution. Rabbit antiserum against mouse cathepsin D was kindly provided by Dr. Regina Pohlmann (Westfälische Wilhelms-Universität Münster, Germany). The rat monoclonal antibody to mouse LAMP-1 (clone 1D4B) developed by J. Thomas August (Johns Hopkins University, Baltimore, MD) was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences (University of Iowa, Iowa City, IA). Affinity-purified goat antibodies to recombinant mouse cathepsin D (R&D Systems, Minneapolis, MN) and a C-terminal CREG peptide (Santa Cruz Biotechnology, Santa Cruz, CA) as well as the mouse monoclonal antibody to protein-disulfide isomerase (Stressgen Bioreagents, Vancouver, Canada) were purchased from commercial suppliers.

Cell culture

The following cell lines were propagated in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at