Oligomerization State-dependent Activation of NF-κB Signaling Pathway by Adipocyte Complement-related Protein of 30 kDa (Acrp30)*

Received for publication, May 21, 2002, and in revised form, June 24, 2002
Published, JBC Papers in Press, June 28, 2002,
DOI 10.1074/jbc.C200312200

Tsu-Shuen Tsao‡§, Heather E. Murrey‡, Christopher Hug‡§, David H. Lee‡§, and Harvey F. Lodish***

From the Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142, ‡Division of Respiratory Diseases, Children’s Hospital, Boston, Massachusetts 02115, and **Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Adipocyte complement-related protein of 30 kDa (Acrp30)/adiponectin is an adipocyte-derived hormone that affects lipid and glucose metabolism in muscle and liver, but its physical and biochemical properties are poorly characterized. Here we have used several approaches to show that Acrp30 expressed in and purified from Escherichia coli and human embryonic kidney 293T cells forms trimers and hexamers; 293T cells also produce a higher molecular weight species. Similar Acrp30 oligomers were found in mouse serum as well as in 3T3-L1 adipocyte-conditioned medium, although in different proportions. In parallel, we assessed whether Acrp30 is a signaling molecule by searching for promoter or enhancer elements that respond to Acrp30 or its isolated trimeric globular C-terminal domain, gAcrp30. Acrp30 addition to C2C12 myocytes or myotubes led to activation of NF-κB transcription factor in a manner dependent upon phosphorylation and degradation of IκB-α. Importantly, only hexameric and larger isoforms of Acrp30 activated NF-κB; trimeric Acrp30 or gAcrp30 could not activate NF-κB. Our data indicate that oligomerization of Acrp30 is important for at least some of its biological activities, and changes in the relative abundance of each oligomeric isoform in plasma may regulate Acrp30 activity.

Adipocyte complement-related protein of 30 kDa (Acrp30), 1

* The work on Acrp30 in the laboratory of H. F. L. was supported by National Institutes of Health Grant R37DK47618 and by a grant from Genset Corp. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

‡ Supported by fellowships from the Ares-Serono Foundation and the American Diabetes Association.

§ A Howard Hughes Medical Institute Fellow of the Life Sciences Research Foundation.

II To whom correspondence should be addressed: Whitehead Inst., 9 Cambridge Center, Cambridge, MA 02142. Tel.: 617-258-5216; Fax: 617-258-6768; E-mail: lodish@wit.mit.edu.

1 The abbreviations used are: Acrp30, adipocyte complement-related protein of 30 kDa; gAcrp30, globular Acrp30; HMW, high molecular weight; DME, Dulbecco’s modified Eagle’s; HEK, human embryonic kidney; PBS, phosphate-buffered saline; BSS, bis(2-sulfosuccinimidyl) suberate; LPS, lipopolysaccharide.

Known also as adiponectin, is a circulating hormone secreted only by adipocytes (1–3). Its structural features include an N-terminal collagenous region containing 22 Gly-X-Y repeats and a trimeric C-terminal globular domain whose structure resembles the trimeric β-stranded jellyroll topology of tumor necrosis factor-α (4). Acrp30 expression and serum concentrations are reduced in a variety of obese and insulin-resistant states in humans, monkeys, and mice (5, 6). Conversely, weight loss, caloric restriction, or thiazolidinedione treatment increases Acrp30 levels in humans and mice (5, 6). Injection of full-length Acrp30 or its isolated globular portion (gAcrp30) lowers serum glucose and free fatty acid levels in mice (7, 8). Long term treatment with Acrp30, and more potently gAcrp30, reduces body weight in high fat-fed obese mice (7) and improves insulin action in insulin-resistant mice (9). These effects are accompanied by increased rate of fatty acid oxidation and decreased triglyceride content in muscle (7, 9). In contrast, full-length Acrp30 but not gAcrp30 enhances the ability of insulin to suppress gluconeogenesis and glucose release by primary hepatocytes (8).

Given the metabolic functions of Acrp30 and its potential involvement in the development of insulin resistance, remarkably little is known of its biochemical and physical properties. Analysis of human and mouse plasma by size exclusion gel chromatography or sucrose velocity gradient revealed that Acrp30 forms multiple species of different apparent molecular weights (1, 10, 11). A portion of the high molecular weight Acrp30 in plasma has been isolated by gelatin-cellulose chromatography (10, 11), possibly due to an affinity for collagen.

Here we have used several approaches to show that Acrp30 expressed in and purified from Escherichia coli and human embryonic kidney 293T cells forms stable trimers and hexamers; 293T cells also produce a diffuse higher molecular weight species. Similar Acrp30 oligomers were found in mouse serum as well as in 3T3-L1 adipocyte-conditioned medium. Whereas the most abundant Acrp30 species in serum are HMW and hexamers, those secreted from 3T3-L1 adipocytes are mostly trimers. We also show that Acrp30 is a hormone: addition of Acrp30 to C2C12 myocytes or myotubes led to activation of the NF-κB transcription factor in a manner dependent upon phosphorylation and degradation of IκB-α. Importantly, only hexameric and larger isoforms of Acrp30 activated NF-κB; trimeric Acrp30 or gAcrp30 could not. Although the biological significance of NF-κB activation is unclear, these results suggest that activity of Acrp30 may be controlled at least partially by changes in the relative distribution of the different oligomeric isoforms.

EXPERIMENTAL PROCEDURES

Cell Line Generation and Maintenance—C2C12 cells (ATCC) were maintained in DME medium supplemented with 10% fetal calf serum. Differentiation was initiated by changing to 2% horse serum after reaching confluency. 3T3-L1 fibroblasts were maintained and differentiated into adipocytes as described previously (12). C2C12 cells expressing IκB-α with S32A and S36A substitutions were generated as described previously (12).

Production and Gel Filtration Analysis of Acrp30 from E. coli and Human Embryonic Kidney (HEK) 293T Cells—E. coli strain BL21 was

protein of 30 kDa; gAcrp30, globular Acrp30; HMW, high molecular weight; DME, Dulbecco’s modified Eagle’s; HEK, human embryonic kidney; PBS, phosphate-buffered saline; BSS, bis(2-sulfosuccinimidyl) suberate; LPS, lipopolysaccharide.
transformed with pTRCHis vector (Invitrogen) containing a His6 tag, human rhinovirus 3C protease recognition site, and either Acrp30 cDNA residues 18–247 (full length) or 107–247 (gAcrp30). Expression was induced with isopropyl-1-thio-β-D-galactopyranoside for 50 min at 37 °C. Following centrifugation and sonication, cell lysates were filtered and bound to nickel-agarose beads for 1 h at 4 °C and eluted with increasing concentrations of imidazole. Fractions containing pure Acrp30 were pooled and treated with PreScission protease (Amersham Biosciences) at 4 °C overnight. PreScission protease and cleaved His6 tag were removed by glutathione-Sepharose and nickel-agarose beads, respectively. Acrp30 secreted from HEK cells was purified as described previously (8) except cells were transiently transfected with mouse Acrp30 cDNA (residues 1–247) in pcDNA3.1 (Invitrogen). Following dialysis against PBS, purified protein samples were fractionated in a 16/60 Superdex 200 column (Amersham Biosciences) and eluted with PBS.

Analysis of Acrp30 from Mouse Serum and 3T3-L1 Adipocyte-conditioned Medium—Two milliliters of mouse serum (Sigma) were loaded onto a 16/60 Superdex 200 gel filtration column and eluted with PBS. Fractions (0.6 ml) were collected, and the amount of Acrp30 in each fraction was analyzed by Western blot analysis using an antisera directed against an epitope in the globular domain (SVGLETRTVPN-VPRFTK) of mouse Acrp30. 3T3-L1 adipocytes differentiated for 8 days were washed twice with serum-free DMEM medium and incubated over-night with serum-free DMEM medium plus 0.05% bovine serum albumin. Conditioned medium was centrifuged and filtered prior to gel filtration and immunoblot analysis.

Equilibrium Sedimentation—Samples were centrifuged at 8000 rpm for 18 h at 10 °C in a Beckman XL-A analytical ultracentrifuge before absorbance was recorded. Data were fit globally, using MacNolin PPC (13), to the following equation that describes sedimentation of a homogeneous species: 

$$
\text{Abs} = B + A' \exp[(M / \tau^2) (x^2 - x_0^2)],
$$

where Abs = absorbance at radius x, A' = absorbance at reference radius x, H = (1 - \nu_0^2)/2RT, R = gas constant, T = temperature in Kelvin, \nu = partial specific volume = 0.7189613 ml/g, \rho = density of solvent = 1.0061 g/ml, \omega = angular velocity in radians/s, M = apparent molecular weight, and B = absorbance (blank).

Cross-linking of Purified Acrp30 Oligomers—Individual peaks of Acrp30 eluted from the gel filtration column in Fig. 1 were collected, and different concentrations (0.1, 1, and 10 mg/ml) of bis(sulfosuccinimidyl) suberate (BS3, Pierce) were added to each Acrp30 species (40 µg/ml) for 30 min at room temperature. Reactions were quenched by a 15-min incubation at room temperature with 50 mM Tris at pH 8.0. Western blot analysis was performed as described above.

Lysate Reporter Assay—C2C12 cells were co-transfected with plasmids encoding firefly luciferase under the control of E-selectin promoter (14) and β-galactosidase driven by the cytomegalovirus promoter using FuGENE 6 (Roche Molecular Biochemicals). Following overnight or 6-h incubation with Acrp30, gAcrp30, or LPS, cells were washed, and luciferase activity was assayed.

RESULTS

As judged by size exclusion chromatography and sucrose gradient analysis serum Acrp30 is mainly found in high molecular weight complexes (1, 10, 11), although their precise composition is uncharacterized. We therefore determined the multimerization state of Acrp30 and its isolated globular domain, gAcrp30, produced in E. coli. To this end full-length Acrp30 was expressed and purified from E. coli as a His6-tagged protein precursor that was subsequently treated with PreScission pro-

2 MacNolin PPC can be found at www.cauma.uthscsa.edu/software/
FIG. 2. Oligomerization states of distinct Acrp30 species purified from E. coli. A, representative sedimentation equilibrium trace of the apparent 410-kDa Acrp30 isoform (Hexamer) purified from E. coli. Initial protein concentration was 3 μM in 5.7 mM phosphate (pH 7.5), 137 mM NaCl, 2.7 mM KCl. The random residuals (top panel) indicated a good fit to a single ideal-species model. B, sedimentation equilibrium trace of the 209-kDa Acrp30 (Trimer A) species from E. coli. C, elution profile of the globular domain of Acrp30 (gAcrp30) in gel filtration chromatography. mAU, milliabsorbance unit.

3A) since the molecular mass of the largest cross-linked species equaled that of about six Acrp30 polypeptides. Similar results were obtained by subjecting the E. coli-produced hexamer (Fig. 2A) to cross-linking (Fig. 3B). Similarly, the 226-kDa species (Trimer A) secreted by HEK cells was a trimer (Fig. 3A), and similar results were obtained by subjecting the E. coli-produced Trimer A and B species to cross-linking (Fig. 3B). While we could not purify enough Acrp30 from mouse serum or 3T3-L1 adipocytes to perform biophysical characterization, the Western blots summarized in the top right and bottom right panels of Fig. 1 indicate that these too contain trimer A, hexamer, and HMW Acrp30 species, although in different proportions.

To determine whether Acrp30 can affect intracellular signaling pathways, we conducted a systematic search of cis-acting enhancer or promoter elements whose transcriptional activation reflects stimulation of various signaling pathways. Of many luciferase reporter constructs tested in C2C12 myoblasts, only expression from the NF-κB-responsive E-selectin promoter (14) was increased significantly by Acrp30 purified from E. coli (Fig. 4A). Notably, gAcrp30 had no effect. Reporter constructs that contained glucocorticoid, heat shock, serum, AMP, p21, and p38 response elements were unaffected either by Acrp30 or gAcrp30 addition (data not shown). Fig. 4B shows that activation of the E-selectin NF-κB-responsive promoter was correlated with release of NF-κB from the inhibitory binding protein IκB-α. Treatment of undifferentiated C2C12 cells for 30 min with Acrp30 (but not gAcrp30) resulted in marked phosphoryylation of IκB-α at Ser-32 as well as degradation of IκB-α (Fig. 4B). By 2 h the steady-state levels of IκB-α returned to normal even though increased phosphorylation persisted. Experiments using differentiated C2C12 cells yielded similar results (data not shown). Acrp30 failed to elicit increased luciferase reporter expression from the E-selectin promoter in C2C12 cells expressing a dominant negative IκB-α, one containing serine to alanine substitutions at positions 32 and 36 (data not shown). This indicates that activation of NF-κB by Acrp30 depends upon phosphorylation and degradation of IκB-α.

Fig. 5A shows that only hexameric and larger forms of Acrp30, purified either from E. coli or HEK cells, transcriptionally activated the NF-κB-responsive E-selectin promoter; Trimer A (and Trimer B, data not shown) forms had negligible effects. Acrp30 HMW and hexamer isoforms activated NF-κB in a dosage-dependent manner; 50% maximal activation of the E-selectin promoter was achieved by 3.5 nM Acrp30 hexamer or 2 nM trimer equivalent of HMW Acrp30 produced by transfected 293T cells (data not shown), a concentration expected of typical receptor-ligand interactions. Consistent with these findings, Acrp30 hexamers induced phosphorylation and degradation of IκB-α in C2C12 cells (Fig. 5B). Although the Trimer A Acrp30 isoform did not cause degradation of IκB-α, it was able to increase phosphorylation of IκB-α at Ser-32 (Fig. 5B), indicating that it is able to partially activate the NF-κB signaling pathway.

DISCUSSION

The major result of our study was that Acrp30 hexamer and HMW species but not Acrp30 or gAcrp30 trimers cause activation of NF-κB in C2C12 myoblasts and differentiated myotubes. One puzzle concerning the potential role of Acrp30 as a genuine endocrine hormone is its high circulating concentration. The mean plasma Acrp30 concentration is ~10 μg/ml (5). This is equivalent to a 130 nM concentration of the basic Acrp30 trimer (based upon monomer molecular mass of 25 kDa). Thus, as compared with other metabolic hormones like insulin and glucagon, the concentration of Acrp30 in circulation is extremely high. Multimerization represents one potential mechanism to effectively reduce the concentration of the active form of Acrp30 (6, 8). Indeed our results suggest that one way to regulate activity of Acrp30 is through its multimerization state.

The biological significance of NF-κB activation by Acrp30 is
Acrp30 Multimerization and NF-κB Activation

FIG. 5. Activation of NF-κB by different Acrp30 isoforms. A, activation of an NF-κB-responsive promoter in undifferentiated C2C12 cells following incubation with 4 μg/ml Acrp30 HMW species produced in transfected 293T cells, hexamer produced in transfected HEK 293T or E. coli cells, or Trimer A isoforms purified from E. coli or HEK cells. Similar results were obtained after only 4 h of incubation with Acrp30 hexamer and Trimer A (data not shown). B, Ser-32 phosphorylation (upper panel) and degradation (lower panel) of IκB-α in C2C12 cells following treatment with either 4 μg/ml Acrp30 hexamer or Trimer A produced in E. coli.

currently under investigation. Full-length Acrp30, but not gAcrp30, increases the ability of insulin to suppress hepatic glucose production (8, 16). This pattern is consistent with activation of NF-κB by hexameric and larger forms of Acrp30 but not by trimeric Acrp30 or gAcrp30. However, there is no published evidence linking NF-κB activation to hepatic glucose production. In addition, a recent study suggests that Acrp30-mediated inhibition of hepatocyte glucose production is related to the hydroxylation and glycosylation of the four conserved lysine residues in the Acrp30 collagen domain (15). Hydroxylation- and glycosylation-deficient Acrp30, produced in E. coli cells, has been reported to exhibit a decreased ability to inhibit hepatocyte glucose production (15). Activity of the E. coli-produced protein was equal to that of a mutant Acrp30, whose four lysine residues in the collagen domain were substituted with arginines, that was produced in transfected mammalian cells (15). In contrast, we showed here that NF-κB activation depends only on the oligomerization state of Acrp30, not on its source or on any posttranslational modifications not made in bacterial cells. Since Acrp30 hexamer produced in either HEK or E. coli cells activates NF-κB, lysine hydroxylation and glycosylation of Acrp30 most likely does not contribute to NF-κB activation. Current data, however, do not preclude the possibility that NF-κB activation is one of multiple signaling pathways required for Acrp30-mediated inhibition of hepatocyte glucose production.

It is noteworthy that the predominant isoform of Acrp30 secreted by 3T3-L1 adipocytes is Trimer A (Fig. 3A). Perhaps the formation of the hexamer and HMW species occurs only after the protein reaches the circulation. Only Acrp30 hexamers and larger oligomers cause activation of NF-κB; Acrp30 trimers are inactive as is gAcrp30. Thus clustering of cell surface receptors by multimers of Acrp30 trimers may effect signal transduction in a manner unattainable by gAcrp30 or Acrp30 trimers.

In cultured cells 50% activation of an NF-κB-responsive promoter is achieved at 3.5 nM Acrp30 hexamer or 2 nM trimer equivalent of HMW species, while the serum concentration of Acrp30 is much higher (about 50–100 nM trimer equivalent). Also, the isoforms of Acrp30 that can activate NF-κB, hexamer and HMW, are also those that are the most abundant in serum. However, movement of Acrp30 from the circulation to muscle or liver cells may be impeded by collagen-binding proteins in the endothelium and in other extracellular matrices. Indeed, recombinant Acrp30 binds tightly to collagens I, III, and V (17). If removal of Acrp30 from circulation is a regulated process, the actual Acrp30 concentration seen by muscle or liver cells may well be very small.

In summary, we have identified three distinct and stable isoforms of Acrp30 produced both by E. coli and cultured mammalian cells and in mouse serum: trimer, hexamer, and an even higher molecular weight form. Because of its ability to lower both blood glucose and free fatty acids, Acrp30 and/or gAcrp30 shows promise as a therapeutic agent for diabetes and obesity. Identifying the different cell surface receptors for these proteins will be necessary to determine how Acrp30 but not gAcrp30 leads to activation of the NF-κB pathway. It may also tell us the biological significance of NF-κB activation by Acrp30. In the future, it will be important to determine whether alterations in the relative distribution of different Acrp30 isoforms can regulate Acrp30 activity.

Acknowledgments—We thank J. Hancock for expert technical support and Drs. J. Bogan, L. J. S. Huang, and L. Rezende for valuable discussions throughout this study.

REFERENCES
1. Scherer, P. E., Williams, S., Fogliano, M., Baldini, G., and Lodish, H. F. (1995) J. Biol. Chem. 270, 26746–26749
2. Hu, E., Liang, P., and Spiegelman, B. M. (1996) J. Biol. Chem. 271, 10697–10703
3. Maeda, K., Okubo, K., Shimomura, I., Funahashi, T., Matsuura, Y., and Matsuura, K. (1996) Biochem. Biophys. Res. Commun. 221, 286–289
4. Shapiro, L., and Scherer, P. E. (1998) Curr. Biol. 8, 335–338
5. Tsao, T. S., Lodish, H. F., and Fruebis, J. (2002) J. Biol. Chem. 277, 213–221
6. Berg, A. H., Combs, T. P., and Scherer, P. E. (2002) Trends Endocrinol. Metab. 13, 84–89
7. Fruebis, J., Tsao, T. S., Javorschi, S., Ebbets-Reed, D., Erickson, M. R., Yen, F. T., Bihain, B. E., and Lodish, H. F. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 2065–2070
8. Berg, A. H., Combs, T. P., Du, X., Brownlee, M., and Scherer, P. E. (2001) Nat. Med. 7, 947–953
9. Yamauchi, T., Komon, J., Waki, H., Terauchi, Y., Kubota, N., Harata, K., Mori, Y., Ide, T., Murakami, K., Tsukabaya-Kasaoa, N., Enoki, O., Akanuma, Y., Gavrilova, O., Vinson, C., Reitman, M. L., Kagechika, H., Shudo, K., Yoda, M., Nakano, Y., Toke, K., Nagai, R., Kimura, S., Tomita, M., Frugel, P., and Kadowaki, T. (2001) Nat. Med. 7, 941–946
10. Yoda, M., Nakano, Y., Toke, T., Shioda, S., Choi-Miura, N. H., and Tomita, M. (2001) Int. J. Obes. Relat. Metab. Disord. 25, 75–83
11. Nakano, Y., Toke, T., Choi-Miura, N. H., Maeda, T., and Tomita, M. (1996) J. Biochem. (Tokyo) 120, 803–812
12. Ruan, H., Horkoben, N., Golu, T. R., Van Paria, J., and Lodish, H. F. (2002) Diabetes 51, 1319–1336
13. Johnson, M. L., Correia, J. J., Yphantis, D. A., and Halvorson, H. R. (1981) Biophys. J. 36, 575–588
14. Schindler, U., and Baichwal, V. R. (1994) Mol. Cell. Biol. 14, 5820–5831
15. Wang, Y., Xu, A., Knight, C., Xu, L. Y., and Cooper, G. J. (2002) Diabetes 51, 1319–1336
16. Okamoto, Y., Arita, Y., Nishida, M., Muraguchi, M., Ouchi, N., Takahashi, M., Iwata, T., Inui, Y., Kihara, S., Nakamura, T., Yamasita, S., Miyagawa, J., Funahashi, T., and Matsuzawa, Y. (2000) Horm. Metab. Res. 32, 47–50