Vol. 170, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1990 Pages 153-161

HUMAN PLATELET GLYCOPROTEIN V: A SURFACE LEUCINE-RICH GLYCOPROTEIN RELATED TO ADHESION

Gerald J. Roth*†, Todd A. Church*, Brad A. McMullen** and Stuart A. Williams*

*Hematology Section, Medical and Research Services,
Veterans Administration Medical Center, Seattle, Washington 98108

Departments of Medicine and **Biochemistry,
University of Washington, Seattle, Washington 98195

Received May 18, 1990

Human platelet glycoprotein V (Mr 82,000) is a surface glycoprotein and a substrate for thrombin, undergoing proteolytic cleavage by thrombin and releasing a soluble fragment, glycoprotein Vfl (Mr 69,000). It does not appear to be the receptor for thrombin's agonist effect on platelets. A congenital platelet disorder, Bernard-Soulier syndrome, is marked by a deficiency of glycoprotein V and two other surface glycoproteins, Ib-IX. The latter two, Ib-IX, constitute the platelet receptor for von Willebrand factor, mediate arterial platelet adhesion, and contain unique 24-amino acid sequences, termed "leucine-rich glycoprotein" segments. The segments relate to adhesive function and distinquish the leucine-rich glycoprotein family. Surface glycoprotein V is not physically associated with Ib-IX nor does it bind to von Willebrand factor. To date, no common denominator has been found that explains the combined deficiency of glycoproteins V and Ib-IX in Bernard-Soulier syndrome. This study describes the isolation of glycoprotein V/anti-glycoprotein V antibody and the analysis of three glycoprotein V peptides that contain"leucine-rich" sequences. Therefore, glycoprotein V shares the "leucine-rich" structure with platelet glycoproteins Ib-IX and belongs to the family of leucine-rich glycoproteins.

Human platelet glycoprotein(GP) V was originally described in 1977 as a single-chain, surface glycoprotein (approximate Mr 82,000), detectable by surface labelling techniques.[1] The protein was subsequently purified from the conditioned media of washed, intact platelets that had been suspended in neutral salt-containing buffer and incubated overnight.[2] The work suggested that glycoprotein V was a peripheral membrane protein, released from the platelet surface by salt-extraction under mild conditions. Later studies indicate that

†To whom correspondence should be addressed: Seattle VAMC (111), 1660 S. Columbian Way, Seattle, WA 98108.

Abbreviations: GP-glycoprotein, vWf-von Willebrand factor, LRG-leucine-rich glycoprotein, HPLC-high pressure liquid chromatography.

0006-291X/90 $1.50

"extraction" is possibly mediated by calpain proteolysis, implying that glycoprotein V may be a typical membrane-bound protein with a discrete transmembrane domain.[3,4] Other structural features of glycoprotein V include a near-neutral isoelectric point (pI 5.85-6.55),[2] a high leucine content,[5] and a large amount of carbohydrate that constitutes approximately one-third of glycoprotein's weight.[2,5] Glycoprotein V also provides antigenic determinants on the platelet surface for anti-platelet antibodies.[6]

In terms of function, thrombin cleaves glycoprotein V, releasing a soluble fragment termed glycoprotein V_{fl} (Mr 69,000).[1,2,7] As the only known thrombin substrate on the platelet surface, glycoprotein V is a logical candidate to be the thrombin receptor. However, direct experiments show that platelets lacking glycoprotein V (on a congenital,[8] proteolytic[9] or immunologic[3] basis) respond to thrombin in a nearly normal fashion. The physical state of glycoprotein V is not closely correlated with the agonist effect of thrombin.[10] The observations imply that glycoprotein V may influence the effect of thrombin on platelets, but the protein is not likely to be the actual thrombin receptor. A unique NH_2-terminal amino acid sequence of a GP V thrombin fragment has been reported.[5]

A congenital platelet disorder, Bernard-Soulier syndrome,[11] is marked by the deficiency of surface glycoprotein V[12] and two other surface glycoproteins, Ib and IX.[12-14] The latter two form a non-covalent complex, the GP Ib-IX complex, on the platelet membrane[15] and provide the receptor for von Willebrand factor (vWf) that mediates platelet adhesion under conditions of rapid blood flow.[16,17] However, glycoprotein V is not involved directly in platelet adhesion and is not physically associated with the glycoprotein Ib-IX complex. The common denominator among the three proteins that leads to the combined deficiency state in Bernard-Soulier syndrome is unknown. Perhaps, all three proteins (Ib,V,IX) must participate in some or all of the steps (synthesis, processing, assembly) that lead to the expression of the mature proteins on the platelet surface.

The primary structure of the polypeptides of the GP Ib-IX complex (Ibalpha/Ibbeta,IX), determined by direct sequencing and cDNA cloning/characterization,[18-21] is marked by the presence of unique 24-amino acid, leucine-rich glycoprotein (LRG) segments that distinquish a family of proteins, the leucine-rich glycoproteins.[18-22] The function of the LRG segment is unknown but may involve adhesion events between cells or between cells and matrix components. Currently, the primary structure of glycoprotein V is unknown, and neither complimentary nor genomic DNA encoding glycoprotein V has been described. In the current study, glycoprotein V and anti-glycoprotein V antibody have been isolated, and fragments of the protein have been sequenced, revealing the presence of leucine-rich glycoprotein segments.

METHODS

Starting material for glycoprotein V purification consists of platelet-rich plasma (approx. 500 ml, 13 mM citrate, 10% platelets-w/v) obtained by continuous flow centrifugation (platelet pheresis) of volunteer donors with essential thrombocythemia (platelet counts $1-3x10^6/mm^3$). For assay of glycoprotein V, proteins are incubated in the presence or absence of thrombin (5.0 units/ml-provided by John Fenton,Albany NY, 90 min, 22°), separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and stained with Coomassie blue.[23] Glycoprotein V is detected as a protein (Mr 82,000) that is reduced in size by thrombin proteolysis, yielding glycoprotein V_{fl} (Mr 69,000).[2]

Following addition of 5mM EDTA, platelets are collected by centrifugation (5,000xg,15 min,22°), resuspended in buffer (1.5 l, 10mM Hepes, pH 7.6, 0.15M NaCl, 1mM EDTA) and recentrifuged. The washed fresh platelets (<4 hours post-pheresis) are suspended in the same buffer (2.0 l) containing 0.3 M NaCl, incubated overnight (37°C,16 hrs), and centrifuged (25,000xg,45min,4°C). Later steps are carried out at 4°C. From the supernate, an NH_4SO_4 precipitate (37.5-52.5% saturation) is collected, solubilized in 10 ml 50mM KH_2/K_2HPO_4, pH 6.8, 1 mM EDTA, dialysed against the same buffer and applied to a column (2.5x100 cm) of Sephacryl S-200 (Pharmacia) equilibrated in the same buffer. Eluted fractions containing glycoprotein V are pooled, dialysed against 5mM KH_2/K_2HPO_4, pH 6.8, 0.2 mM EDTA, applied to a column (1.5x10 cm) of hydroxylapatite equilibrated in the same buffer and eluted by a linear gradient (5 to 200 mM KH_2/K_2HPO_4, pH 6.8, 0.2 mM EDTA, 250 ml total volume). Fractions containing glycoprotein V are pooled, dialysed against 20 mM NaH_2/Na_2HPO_4, pH 7.5, 0.2 mM EDTA and applied to a column (0.9x5 cm) of DEAE-cellulose equilibrated in the same buffer. Non-adsorbed ("pass-through") protein-containing fractions are pooled, dialysed against 10 mM NH_4HCO_3, lyophilized, dissolved in 1 ml 0.1 M Tris-acetate, pH6.8 and injected on an HPLC gel filtration column (2.1x60 cm, TSK4000) equilibrated in the same buffer. Fractions containing glycoprotein V, Mr 82,000, are pooled, dialysed against 10 mM NH_4HCO_3, lyophilized and rechromatographed on the TSK 4000 column, providing the final preparation of purified glycoprotein V.

Rabbits were immunized with pure glycoprotein V in Freund's adjuvant. Affinity-purified, anti-glycoprotein V antibody was isolated on a glycoprotein V-Sepharose column, labeled with ^{125}I, and analysed for specificity by Western blotting using whole platelet lysates as antigen. The techniques have been described.[24,25]

Purified glycoprotein V was reduced, pyridylethylated with 4-vinylpyridine,[26] treated with CNBr and fractionated by reverse-phase HPLC. Two broad peaks were eluted, pooled, reconcentrated, digested with trypsin, applied to a C_{18} reverse-phase HPLC column, and eluted with an acetonitrile gradient. Multiple peaks were observed in the chromatogram of the column eluate, and proteins in discrete peaks were collected and subjected to amino acid sequence analysis using a Model 477A Protein Sequencer with an on-line HPLC Model 120A Analyser (Applied Biosystems).[20,21]

RESULTS AND DISCUSSION

The methodology employed for glycoprotein V purification follows that described by Berndt and Phillips[2] except for the final HPLC gel filtration step. The large amounts of fresh platelets (50 gm wet weight, 6 gm protein) used as starting material facilitate the purification process. The amount of protein eluted by overnight incubation varied from 200-300 mg with 40-60 mg carried through the NH_4SO_4 precipitation step and 12-18 mg in the fractions from the

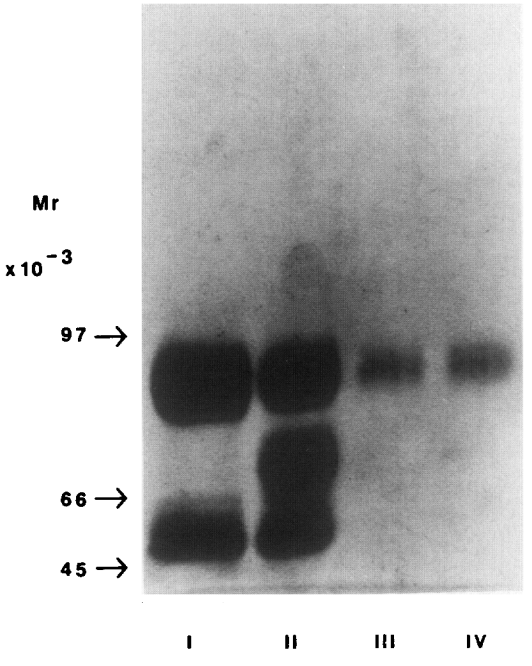

Figure 1. Gel electrophoretic analysis of purified fractions of glycoprotein V.

Partially purified glycoprotein V (10ug) from the DEAE cellulose step was incubated without (lane I) or with (lane II) thrombin (5U/ml,90 min,22°) and fractionated by SDS-PAGE (Laemmli system, 7.5% acrylamide, reduced). Partial digestion occurred. Analysis of the final preparation of purified glycoprotein V from the second HPLC gel filtration step is shown in lanes III and IV (ascending and descending limbs, respectively, of the GP V peak in Fig. 2B). Proteins were stained by Coomassie blue. Molecular weight standards are noted on the left.

Sephacryl column. Protein eluted from the hydroxylapatite column (3-5 mg) contained 15-30% glycoprotein V as estimated by the SDS-PAGE assay of thrombin-cleavable protein, Mr 82,000-69,000). The "pass through" proteins (1 mg) from the DEAE step contain approximately 50% glycoprotein V, Mr 82,000, cleaved by thrombin to glycoprotein V_{f1}, Mr 69,000 as shown in lanes I and II, Figure 1. Material from the DEAE step was fractionated twice by HPLC gel filtration as shown in Figures 2A and 2B, providing the final, highly purified, Mr 82,000 glycoprotein V of lanes III and IV, Fig.1 (ascending and descending limbs, respectively, of the glycoprotein V peak shown in Fig. 2B). The smaller contaminant protein (Mr 50,000) in lanes I and II of Fig. 1 migrates as a larger protein under the non-reduced conditions of the HPLC gel filtration step, Fig. 2, perhaps due to aggregation.

Affinity-purified rabbit antibody to glycoprotein V was analysed for specificity by the Western blot shown in Figure 3. The bulk of the antibody was directed against glycoprotein V since thrombin altered essentially all of the antigen in platelets from Mr 82,000 (intact) to Mr 69,000 (thrombin-cleaved) as shown in lanes 1 and 2, respectively, Fig.3. The result with larger amounts of antigen substantiates the conclusion that the anti-glycoprotein V antibody is largely

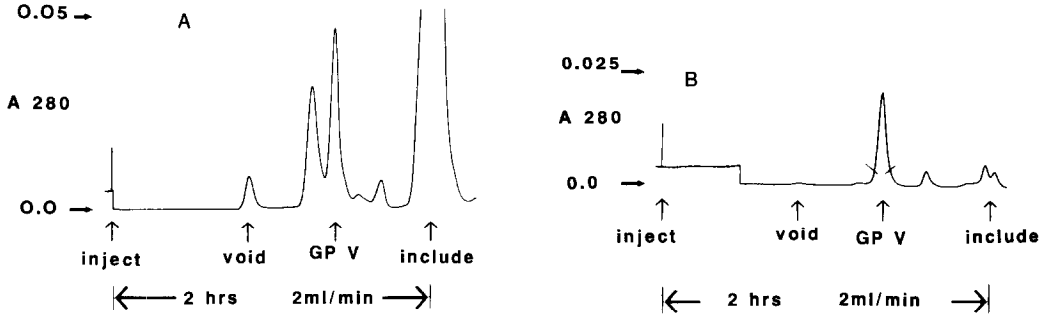

Figure 2. Purification of glycoprotein V by HPLC gel filtration under denaturing conditions.

A. Partially purified glycoprotein V (1mg) from the DEAE step was applied to an HPLC gel filtration column (2.1x60cm, TSK4000, 0.1%SDS, 2ml/min flow) and eluted proteins were detected by absorbance (280nm, 0-0.05 units full scale). Sample injection (inject) and void/GP V/included volume peaks are noted.
B. Protein from the GP V peak (Fig. 2A) was dialysed, lyophilized and rechromatographed as described above (Fig. 2A). Protein in the ascending and descending limbs of the GP V peak were analysed by SDS-PAGE (lanes III and IV, respectively, Fig. 1).

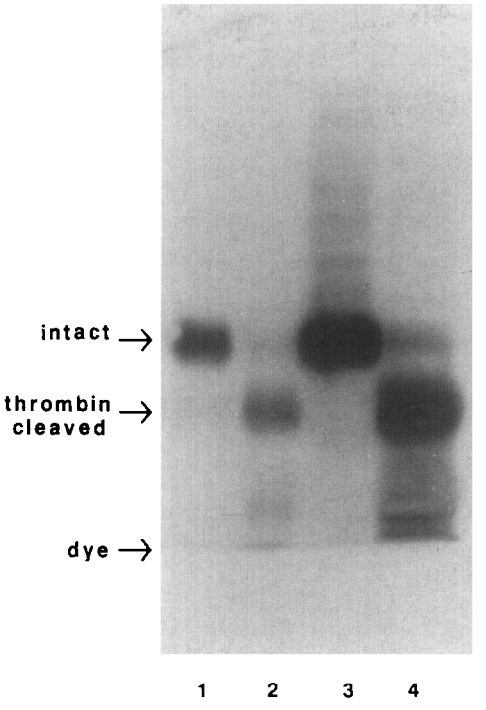

Figure 3. Western blot analysis of anti-GP V antibody.

Saline or thrombin-treated whole platelets ($2x10^9$,-/+ 5u/ml thrombin, 90 min, 22°) were solubilized and proteins separated by SDS-PAGE. Antigens were transferred to nitrocellulose, incubated with ^{125}I anti-GP V, washed and detected by the autoradiogram shown. Lanes 1 (saline) and 2 (thrombin) contain 50 ug platelet protein. Lanes 3 (saline) and 4 (thrombin) contain 250 ug. "Intact"-intact GP V (Mr 82,000), "thrombin cleaved"- GP V_{f1} (Mr 69,000).

mono-specific since the bands corresponding to intact and cleaved GP V intensify in concert with the amount of antigen present (lanes 3 and 4, respectively, Fig. 3). The Western blot analysis of the anti-glycoprotein V antibody identifies the antigen as glycoprotein V, based on the size of the intact protein and its thrombin-cleaved product. The analysis also implies that the antigen was highly purified since its use as an antigen and as an affinity-adsorbant produced a largely mono-specific antibody. The antigen band migrating just above the dye front in lanes 2 and 4, Fig. 3, may correspond to the smaller thrombin fragment of GP V.

Amino acid sequence(s), ranging from 4 to 35 residues in length, were obtained with each of 13 peaks taken from the reverse-phase HPLC fractionation of the

trypsin digest. Both major and minor sequences were found in nine of the samples while single sequences were found in the remaining four. One sequence (GPP?RPAADSSSEAPVHPALAPPSSE?P, see legend for Fig. 4) resembles that reported for a thrombin fragment of glycoprotein V[5], although the two sequences differ inexplicably at positions 3,10,17,18 and 20. Three of the sequences, M 392/393/401, show homology to leucine-rich glycoprotein (LRG) sequences[18-22] when aligned as shown in Figure 4. The individual sequences are continuous as indicated by the arrows (E→L, M392; Q→L, M393; L→L, M401) implying that each of these sequences represents a pair of tandem repeats, consistent with the pattern of tandem repetition of LRG segments in the reported members of this family of proteins. The three pairs of sequences suggest that glycoprotein V contains at least six LRG repeats.

By inspecting the three available GP V sequences as displayed in Figure 4, one can derive the consensus sequence shown (GP V consensus) with conserved residues at various positions within a putative 24-amino acid LRG repeat. In Figure 5, the GP V LRG consensus sequence is compared with those of several human LRG

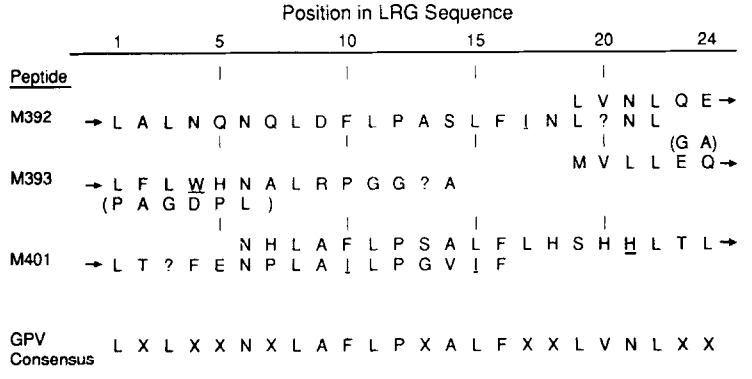

Figure 4. Amino acid sequences of glycoprotein V peptides with homology to leucine-rich glycoprotein (LRG) sequences.

NH_2-terminal amino acid sequences of three individual peptides (M392,393,401) from CNBr/trypsin-treated glycoprotein V are aligned (+) to indicate homology with 24-amino acid, leucine-rich glycoprotein (LRG) sequences (see text, Figure 5 and references 18-22). Arrows denote continuous sequences with the NH_2-terminal portion on an upper line and the COOH-terminal portion on a lower line (peptide M392 example: NH_2-terminal leucine, position 19, L; continuous sequence glutamic acid→leucine, positions 24→1, E→L; COOH-terminal leucine, position 22, L). A minor sequence (GAPAGDPL) was noted in cycles 5-12 of peptide M393. A consensus of the three sequences is given (GP V consensus). The single letter amino acid code is used,(?) indicates an indeterminate residue, (_) an uncertain identification, (X) a non-conserved residue at the given position in the consensus sequence.

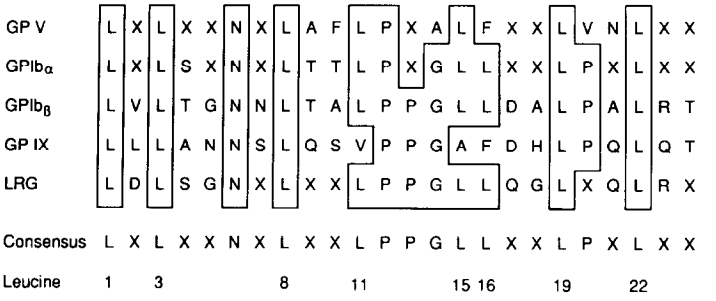

Figure 5. Consensus and single LRG sequences from human platelet and serum proteins.

The consensus LRG sequence from the six available repeats of glycoprotein V (Fig.4) is given along with the consensus LRG sequence from the seven repeats of glycoprotein Ib_{alpha};[18,19] the single LRG sequences from glycoproteins Ib_{beta}[20] and IX;[21] and the consensus LRG sequence from the nine repeats of the "proband" protein, leucine-rich $alpha_2$ glycoprotein of human serum (LRG).[22] An overall consensus of the five upper[2] sequences is given (consensus), conserved residues are enclosed in boxes, and (X) denotes non-conserved residues. The positions of conserved leucines within the 24-amino acid LRG segments are noted (leucine 1,3,8...).

proteins; namely, the three polypeptides of the platelet glycoprotein Ib-IX complex (GP Ibalpha/GP Ibbeta, GP IX) and the serum leucine-rich $alpha_2$ glycoprotein (the "proband" of the LRG family).[22] The sequence homology among these LRG segments is clear-cut, exemplified by the leucines at positions 1,3,8,11,15,19 and 22. These conserved residues distinquish the LRG family and give rise to the "leucine-rich" name. The data in Figures 4 and 5 identify human platelet glycoprotein V as an LRG protein, closely related in this regard to the glycoproteins of the Ib-IX complex. How these LRG segments relate to function or to the common deficiency of glycoproteins Ib,V and IX in Bernard-Soulier syndrome remains to be determined.

ACKNOWLEDGMENTS

We thank Bea Yancy for assistance with the manuscript and the Pheresis Unit of the Puget Sound Blood Center for providing platelet pheresis specimens. The work was supported by a Merit Review Grant (GJR) from the Veterans Administration and by grant HL 39947 (GJR) from the National Institutes of Health.

REFERENCES

1. Phillips,D.R. and Agin,P.P. (1977) Biochem. Biophys. Res. Comm. 75, 940-947.
2. Berndt,M.C. and Phillips,D.R. (1981) J. Biol. Chem. 256, 59-65.
3. Bienz,D., Schnippering,W. and Clemetson,K.J. (1986) Blood 68, 720-725.
4. Clemetson,K.J., Bienz,D., Zahno,M.-L. and Luscher,E.F. (1984) Biochim. Biophys. Acta 778, 463-469.
5. Zafar,R.S. and Walz,D.A. (1989) Thromb. Res. 53, 31-44.

6. Stricker,R.B. and Shuman,M.A. (1986) Blood 67, 1377-1381.
7. Mosher,D.F., Vaheri,A., Choate,J.J. and Gahmberg,C.G. (1979) Blood 53, 437-445.
8. Jamieson,G.A. and Okumura,T. (1978) J. Clin. Invest. 61, 861-864.
9. McGowan,E.B., Ding,A.H. and Detwiler,T.C. (1983) J. Biol. Chem. 258, 11243-11248.
10. Wicki,A.N. and Clemetson,K.J. (1985) Eur. J. Biochem. 153, 1-11.
11. Bernard,J. and Soulier,J.P. (1948) Sem. Hop. Paris 24, 3217-3223.
12. Clemetson,K.J., McGregor,J.L., Dechavanne,M. and Luscher,E.F. (1982) J. Clin. Invest. 70, 304-311.
13. Nurden,A.T. and Caen,J.P. (1975) Nature 255, 720-722.
14. Nurden,A.T., Dupuis,D., Kunicki,T.J. and Caen,J.P. (1981) J. Clin. Invest. 67, 1431-1440.
15. Coller,B.S., Peerschke,E.I., Scudder,L.E. and Sullivan,C.A. (1983) Blood 61, 99-110.
16. Weiss,H.J., Tschopp,T.B., Baumgartner,H.R., Sussman,I.I., Johnson,M.M. and Egan,J.J. (1974) Amer. J. Med. 57, 920-925.
17. Weiss,H.J., Turrito,V.T. and Baumgartner,H.R. (1978) J. Lab. Clin. Med. 92, 750-762.
18. Titani,K., Takio,K., Handa,M. and Ruggeri,Z.M. (1987) Proc. Natl. Acad. Sci. USA 84, 5610-5614.
19. Lopez,J.A., Chung,D.W., Fujikawa,K., Hagen,F.S., Papayannopoulou,T. and Roth,G.J. (1987) Proc. Natl. Acad. Sci. USA 84, 5615-5619.
20. Lopez,J.A., Chung,D.W., Fujikawa,K., Hagen,F.S., Davie,E.W. and Roth,G.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2135-2139.
21. Hickey,M.J., Williams,S.A. and Roth,G.J. (1989) Proc. Natl. Acad. Sci. USA 86, 6773-6777.
22. Takahashi,N., Takahashi,Y. and Putnam,F.W. (1985) Proc. Natl. Acad. Sci. USA 82, 1906-1910.
23. Laemmli,U.K. (1970) Nature 227, 680-685.
24. Canfield,V.A., Ozols,J., Nugent,D. and Roth,G.J. (1987) Biochem. Biophys. Res. Comm. 147, 526-534.
25. Roth,G.J., Ozols,J., Nugent,D. and Williams,S.A. (1988) Biochem. Biophys. Res. Comm. 156, 931-939.
26. Friedman,M., Krull,L.H. and Cavins,J.F. (1970) J. Biol. Chem. 245, 3868-3871.