

Annexin-1 Is Abnormally Expressed in Fragile X Syndrome: Two-Dimensional Electrophoresis Study in Lymphocytes

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The search for targets of FMRP (the product of *FMR1*, the mutated gene in Fragile X syndrome) has predominantly focused on identifying transcripts that are regulated by this RNA-binding protein. This study introduces the use of two-dimensional gel electrophoresis (2D PAGE) as a novel approach for demonstrating changes in protein synthesis secondary to FMRP deficit. By a standardized 2D PAGE protocol, we studied leukocyte homogenates from 30 males with different patterns of *FMR1* mutation and different levels of FMRP. Samples from these subjects were compared to those of 12 normal control males and eight subjects with other mental retardation-associated conditions (i.e., Rett and Down syndromes). We found an abnormal pattern of a major leukocytic protein, identified by 2D PAGE datasets and immunoblotting as annexin-1 (Anx-1). Anx-1 appeared in subjects with Fragile X as multiple rather than 1-2 spots, at ~37 kd, in the pI 5-7 range. The presence and intensity of this Anx-1 pattern was relatively independent of Anx-1 levels and inversely related to total and high MW FMRP immunoreactivities. Based on the 2D PAGE pattern, without obvious MW change, and on dephosphorylation assays, we concluded that Anx-1's abnormality represents an aberrant posttranslational modification other than phosphorylation.

Comparisons of our data with published cytoskeletal protein 2D profiles suggest that Anx-1 may be abnormally acetylated and, consequently, incapable of establishing appropriate N-terminal protein-protein interactions. In addition to its peripheral anti-inflammatory function, Anx-1 mediates glucocorticoid inhibition of the hypothalamo-pituitary-adrenal axis. As the latter seems to be disrupted in Fragile X syndrome, the reported Anx-1 abnormality could be responsible for some aspects of the Fragile X neurobehavioral phenotype. Our data also emphasize the feasibility of using 2D PAGE for disclosing molecular abnormalities in Fragile X and other genetic disorders.

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KEY WORDS: fragile X; *FMR1*; FMRP; 2D PAGE; leukocytes; annexin-1

INTRODUCTION

Fragile X syndrome (FraX) is the most common hereditary cause of mental retardation, affecting approximately 1 in 4,000 males and 1 in 6,000 females [Kaufmann and Moser, 2000]. The mutation, in most cases, consists of an unstable expansion of a CGG trinucleotide repeat within the 5' untranslated region of the *FMR1* gene [reviewed by Kaufmann and Reiss, 1999]. Based on the size of this expansion, individuals are classified as being normal (5–50 repeats), having premutation (PM) (50–200 repeats), or full mutation (FM) (200–2,000 repeats) alleles. A mixed pattern of *FMR1* mutation in which PM and FM alleles are combined is termed as mosaicism (MOS) [Oberlé et al., 1991; Verkerk et al., 1991; Rousseau et al., 1994; Rousseau et al., 1995]. FM- but not PM-size repeat expansions are associated with hypermethylation

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tion of both the CGG polymorphic region and a CpG island located 250 bp proximal to it, which leads to gene silencing and severe physical and neurologic phenotype, particularly in males [Oostra and Halley, 1995; Kaufmann and Reiss, 1999]. Male individuals with unmethylated or partially methylated FM, or partially methylated PM alleles, are called methylation or atypical MOS [Kaufmann and Reiss, 1999]. Males with FM and complete methylation lack the expression of the *FMR1* product, the Fragile X Mental Retardation Protein (FMRP) [Devys et al., 1993; Feng et al., 1995]. Individuals with any type of mosaicism, as well as females with FM, express variable levels of FMRP [Hagerman et al., 1994; Kaufmann et al., 1999]. Based on correlations between molecular phenotype and clinical manifestations, it has been established that FMRP deficiency is responsible for the neurobehavioral manifestations of the syndrome [Oostra and Halley, 1995; Kaufmann et al., 1999].

FMRP is a member of a family of RNA-binding proteins, which also includes the homologues FXR1P and FXR2P [Siomi et al., 1995; Zhang et al., 1995; Khandjian, 1999], which appear to be involved in protein translation [Jin and Warren, 2000]. Intense efforts have been made recently to identify the specific transcript targets of FMRP. Different approaches have been used, including FMRP-RNA binding assays [Ceman et al., 1999; Sung et al., 2000], RNA differential display methods [Zhong et al., 1999], protein interaction assays [Bardoni et al., 1999], and FMRP localization, trafficking, and interaction analyses [Tamanini et al., 1999a; Tamanini et al., 1999b; Beaulieu, 2000]. These studies have already identified putative RNA targets encoding both known and novel proteins, which include, among others, FXR1P, FXR2P, nucleolin, nuclear FMRP interacting protein (NUFIP), and FMRP itself [Bardoni et al., 1999; Ceman et al., 1999; Sung et al., 2000]. Protein expression profiling by two-dimensional gel electrophoresis (2D PAGE) is a complementary approach, which to our knowledge has not yet been used in FraX research. Two-dimensional PAGE, in association with microsequencing techniques and protein databases, has become a powerful and sensitive strategy for the identification of individual proteins in complex mixtures of cellular or tissular proteins [Hall et al., 1993; Hoogland et al., 1999]. Although 2D PAGE has been extensively used for the study of regulation of gene expression in normal [Black et al., 1986] and diseased [Ehrhart et al., 1988] cells, its application to investigations of genetic and developmental disorders has been limited [Fountoulakis et al., 1999; Lubec et al., 1999; Sugawara et al., 2000]. In the case of FraX, the use of 2D PAGE has an additional advantage. Techniques aimed at detecting differential RNA levels, in the case of FraX, rely on the effect of FMRP as a nucleo-cytoplasmic shuttle on RNA stability or trafficking [Zhong et al., 1999]. In contrast, proteomics has the potential of directly evaluating the role of FMRP in protein synthesis.

For this purpose, we developed a standardized protocol for peripheral leukocyte protein profiling. We applied this protocol to males with FraX as well as to

appropriate normal, developmentally delayed, and mentally retarded controls. The selection of this sample was based on our previous data demonstrating that males with FraX showed a wide range of reduction in FMRP immunoreactivity in leukocytes, while females tend to have relatively high levels [Kaufmann et al., 1999]. Consequently, an exclusively male sample is most suitable for examining events secondary to FMRP deficit. In this article, we report the identification, by 2D PAGE, of an abnormal pattern of expression of a major regulatory protein in leukocyte, annexin-1 (Anx-1) or lipocortin-1, in males with FraX. We hypothesize that this molecular abnormality, which is directly related to FMRP deficiency, plays a role in endogenous glucocorticoid dysfunction in FraX and may contribute to some aspects of the neurobehavioral phenotype of FraX.

MATERIAL AND METHODS

Subjects

Blood samples were obtained from a total of 49 subjects corresponding to the following categories: control males (n=12, mean age 15.8 years, range 3.0–37.0 years), two of them developmentally disabled (ages 4.2 and 5.5 years); males with FraX, diagnosed through standard Southern blot testing, with full mutation (n=19, mean age 8.2 years, range 3.0–25.9 years), and mosaic (n=11, mean age 6.6 years, range 3.8–9.8 years) patterns; Rett Syndrome (all female, n=5, mean age 6.8 years, range 2.8–10.4 years); and Down Syndrome (n=3, two males ages 14 and 18 years, one 32-year-old female). The latter two groups were introduced for determination of the specificity of the 2D PAGE patterns. Informed consent for the procedure was obtained from the subjects or legal guardians. Table I and Table II provide information about all of the participating subjects.

Sample Preparation

Protein analyses were done on lymphocyte-enriched samples. Peripheral leukocytes were separated using the CPT-Vacutainer system, which enriches the sample to approximately 80% lymphocytes [Mole et al., 1994]. Subsequent processing was done as described in an earlier study [Kaufmann et al., 1999]. Cells were homogenized in either denaturing buffer: 1X Laemmli (8M urea, 2% 10% SDS, β -mercaptoethanol, 10% glycerol, 62.5mM 0.5M Tris-Cl) or Bio-Rad SDS Reducing Buffer without urea.

2D PAGE

For the purpose of developing a standardized protocol for processing leukocyte homogenates, we tested multiple variables in terms of sensitivity and reliability. Among other factors, we determined that non-denaturing conditions were slightly better for displaying a wide spectrum of proteins. Parameters for strip rehydration and isoelectric focusing were also essential for the success of the protocol. In order to preserve the

integrity of FMRP, all experiments were performed with denatured samples [Kaufmann et al., 1999].

1st dimension: sample volumes containing 30 μ g protein (determined by Bradford protein assay) were added to Amersham Pharmacia Biotech rehydration solution (8M urea, 2% CHAPS, bromphenol blue) with DTT and IPG Buffer for a total volume of 125 μ l. Seventy-centimeter Immobiline DryStrips pH 3–10L or pH 4–7L were placed in ceramic IPGphor strip holders containing the sample solution. Strip rehydration and isoelectric focusing were accomplished using the IPGphor system (Amersham Pharmacia Biotech). Twelve-hr rehydration at 150V and 300Vh was subsequently followed by 30 min isoelectric focusing at 500V and 250Vh, 30 min at 1,000V and 500Vh and 1 hr at 8,000V and 8,000Vh.

2nd dimension: after rehydration and isoelectric focusing, strips were equilibrated for 15 min in Amersham Pharmacia Biotech SDS equilibration buffer (50mM Tris-Cl pH 8.8, 6M urea, 30% glycerol, 2% SDS) with DTT. Equilibration was then repeated for 15 min in SDS equilibration buffer with iodoacetamide. Strips were placed on vertical 1.0 mm 12% SDS-polyacrylamide gels and sealed with 0.5% agarose sealing solution (Amersham Pharmacia Biotech). Electrophoresis was performed in standard running buffer at 200V for 50 min using Bio-Rad's Power Pac 300.

Gel Staining

After 2D PAGE, gels were stained using Bio-Rad Laboratories Spyro Ruby protein gel stain to obtain a UV image, and then silver-stained using Bio-Rad Laboratories Silver Stain Plus for preservation and analysis.

Immunoblotting

Quantitation of specific immunoreactivities was performed on the same denatured whole cell homogenates as described before [Kaufmann et al., 1996; Kaufmann et al., 1997; Kaufmann et al., 1999]. Essentially the same protocol was used for samples resolved by 1D PAGE and 2D PAGE. After protein assays, 20–30 μ g of each sample were electrophoresed in 7.5%–12% SDS/polyacrylamide minigels and transferred onto nitrocellulose blots, immunoprobed, and processed by the enhanced chemiluminescence (ECL). For determination of FMRP immunoreactivity (-ir), we used the 1A monoclonal antibody (Chemicon), as previously reported at 1:750 dilution [Kaufmann et al., 1999]. Annexin-1 (Anx-1) was detected by the monoclonal antibody A13920 (clone 29) (Transduction Laboratories), raised against human endothelium-origin Anx-1, at 1:1,000 dilution [Emmert-Buck et al., 2000]. Standardization of FMRP and Anx-1 quantitations and densitometric analyses has been described in earlier studies [Kaufmann et al., 1997; Kaufmann et al., 1999].

Dephosphorylation Assay

Leukocyte homogenates from individuals with FM were subjected to dephosphorylation by incubation

with intestinal alkaline phosphatase, prior to 2D PAGE, according to a protocol kindly provided by Dr. L. Sternberger [Sternberger and Sternberger, 1983]. The gels were subsequently transferred onto blots and incubated with the Anx-1 antibody. We used, as standards for the enzymatic reaction, changes in intensity of immunoreactivity of phosphorylated neurofilaments and non-phosphorylated neurofilaments, detected by the antibodies SMI-31 and SMI-32, respectively, in brain whole homogenates [Kaufmann et al., 2000] incubated simultaneously with the leukocyte samples. Dilution for both antibodies (Sternberger Monoclonals Incorporated) was 1:1,000.

Patterns of Immunoreactivity

Two observers, who were blind to diagnosis, determined the patterns of immunoreactivity for both FMRP and Anx-1. For FMRP, we used criteria applied in a previous investigation (Table I) [Kaufmann et al., 1999]. FMRP type 1, with more prominent high molecular weight (HMW) bands, is seen in subjects with normal and PM alleles and in many females with FM. FMRP type 2, characterized by approximately equal immunoreactivity for the HMW-ir and 70 kd-ir, is found in most male mosaics and in some females with

TABLE I. Characteristics of Fragile X Subjects Under Study

Subject number	Age	FMR1 mutation	FMRP immunoblot pattern ^a	Annexin-1 2D PAGE pattern ^b
100	3.0	FM	3	I
83	3.3	FM ^c	2/3	W
90	3.6	FM	3	N
77	3.8	MOS	2/3	N
92	3.9	FM ^c	2	N
98	3.9	FM	3	I
93	4.1	FM	3	I
28	4.5	FM	2	N
104	4.9	FM ^c	3	I
115	5.0	MOS ^c	2/3	N
88	5.1	MOS	3	I
106	5.1	MOS ^c	2/3	N
41	5.2	FM	3	I
118	5.8	FM	2	W
123	6.1	MOS	3	W
66	6.5	MOS	3	I
114	6.6	MOS ^c	2	W
80	6.6	FM	3	I
81	6.7	FM	3	I
143	7.1	MOS ^d	1	N
133	7.6	MOS	2	I
119	8.0	FM ^c	3	N
132	9.0	FM	2/3	W
22	9.4	MOS	2	N
44	9.5	FM ^c	2	W
47	9.8	MOS	2	W
84	13.0	FM ^c	2/3	I
50	16.3	FM	2	W
94	18.8	FM	3	I
4	25.9	FM	2/3	N

^aSee Materials and Methods text for FMRP-ir pattern description.

^bN, normal; W, weak abnormal; I, intense abnormal.

^cHigh-functioning subjects (FSIQ \geq 60).

^dMosaic with predominance of premutation alleles.

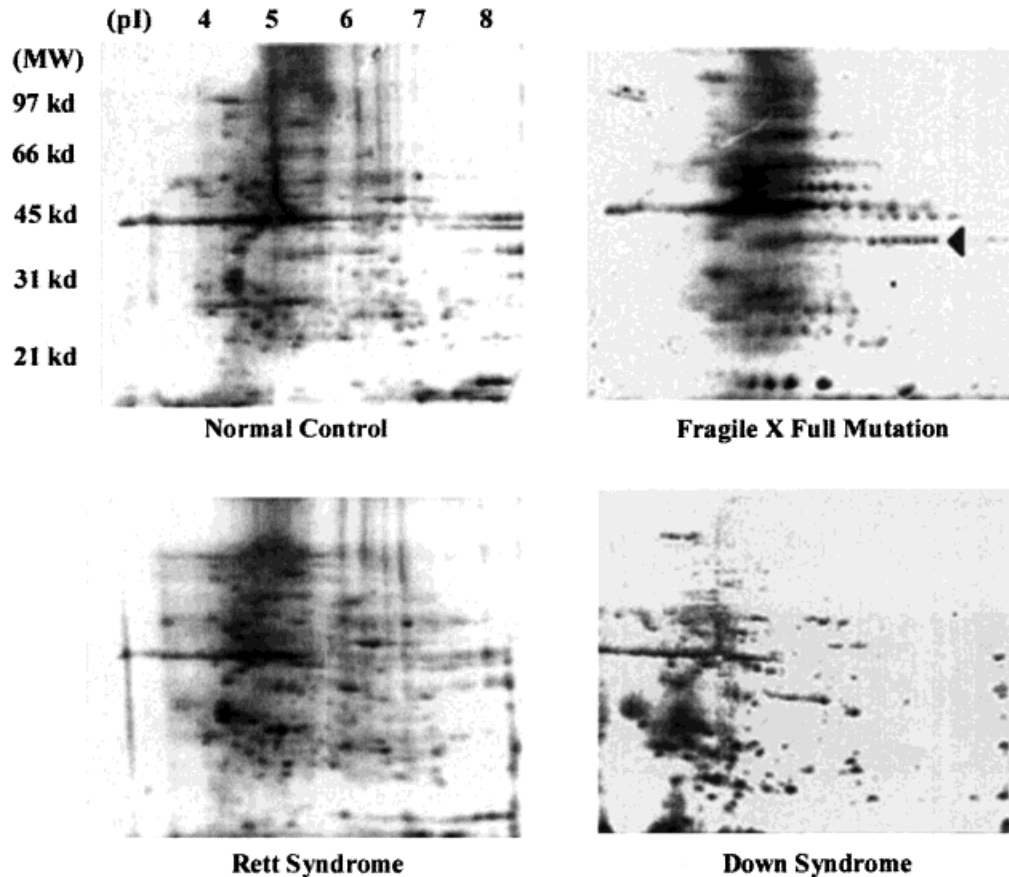


Fig. 1. Patterns of 2D PAGE in leukocyte homogenates from individuals with normal (top left) and full mutation (top right) *FMR1* alleles. The bottom figures represent samples from subjects with Rett and Down syndromes (left and right, respectively). Note the prominent multiple spot

pattern (arrowhead), corresponding to an ~35 kd protein in the pI 5–7 range, seen in the Fragile X subject. Other less consistent groups of spots, which resemble the labeled one, are also found at higher and lower MWs.

FM. In our sample, five male individuals with FM displayed a type 2 FMRP pattern. Type 3, with virtually only 70 kd-ir, is typical of males with FM [Kaufmann et al., 1999]. Males with MOS and minimal FMRP expression showed an intermediate type 2/3 pattern, which was also found in four subjects with FM. Anx-1 patterns were categorized as normal (1–2 spots), weak (4–5 spots), or intense (5–7 spots).

Statistical Analyses

The results of the Kolmogorov-Smirnov tests indicated that FMRP-ir and Anx-1-ir were all distributed normally. Therefore, ANOVA analyses were used for comparing these immunoreactivities among Anx-1 2D patterns. Relative frequency of FMRP-ir and Anx-1 2D PAGE patterns were analyzed by chi-square tests. All statistical analyses were conducted using Statview 5.0[®].

RESULTS

2D PAGE Patterns

In our search for proteins abnormally regulated by FMRP deficit, we examined leukocyte homogenates from 30 males with FraX. The 2D PAGE profiles

revealed an abnormal pattern in 22 subjects, as shown in Figure 1b and Table I. This pattern was found only in one male normal control (Table II), but not in any mentally retarded control (Fig. 1c, d, Table II), and consisted of the presence of several groups of spots characterized by approximately the same MW, but different pIs. Of all of these groups of spots, those found most consistent in FraX subjects corresponded to four to seven spots of an abundant protein characterized by a MW of ~35 kd and a ~pI 5–7 range (Fig. 1b). In both normal and abnormal controls (Fig. 1a, c, d), the protein appears as 1–2 spots at ~ pI 5 at the same MW. The analyses reported here focus exclusively on this abnormal ~35 kd major protein.

The abnormal ~35 kd 2D PAGE pattern was also characterized by variable intensities among FraX subjects. In order to evaluate the relationship between DNA and FMRP patterns on one hand, and this novel aberrant 2D PAGE pattern on the other, we developed a semi-quantitative assessment system. In this procedure, observers blind to diagnosis classified the silver stained 2D PAGE gels as normal, weak, and intense (see Methods). In addition, the weak pattern was associated with less intense gel staining. A full description of the FraX sample, according to this categorical classification, is provided in Table I. In

TABLE II. Characteristics of Control Subjects Under Study

Subject number	Age	Status ^a	2D PAGE Annexin-1 pattern ^b
R1	2.8	RSF	N
27	3.0	CE	N
R3	4.0	RSF	N
38 ^c	4.2	CM	N
31 ^c	5.5	CM	N
33	5.7	CM	N
R5	8.4	RSF	N
R4	8.5	RSF	N
61	8.7	CM	W
R2	10.4	RSF	N
D1	14.0	DSM	N
55	15.0	CM	N
D3	18.0	DSM	N
131	19.0	CM	N
21	20.7	CM	N
130	21.0	CM	N
6	22.6	CM	N
71	26.8	CM	N
D2	32.0	DSF	N
144	37.0	CM	N

^aC, control status; DS, Down syndrome; RS, Rett syndrome; M, male; F, female. All CM showed type 1 FMRP-ir.

^bN, normal; W, weak abnormal.

^cDevelopmentally disabled.

general terms, the most intense 2D PAGE pattern was found among those males with FM while five out of 11 individuals with MOS had a normal pattern (Table I). No age-related differences were found.

Other qualitative abnormalities in leukocyte 2D PAGE patterns were observed in our FraX sample. A generalized reduction in spots, particularly in the < 30 kd, ~pI 5–8 region (Fig. 1b), was noted. This contrasted with a relative increase in spots in the RS and DS homogenates (Fig. 1c, d). A detailed analysis of these additional findings will be the subject of future reports.

FMRP and 2D PAGE Patterns

The first examination of the relationship between FMRP and 2D PAGE patterns consisted of calculating the relative frequency of the three 2D PAGE patterns across the FMRP types [Kaufmann et al., 1999]. Table III presents these analyses, which were circum-

TABLE III. Relationship Between FMRP and Annexin-1 Patterns

FMRP/ pattern	Annexin-1 pattern			Totals
	Normal	Abnormal weak	Abnormal intense	
Type 1	12	1	0	13
Type 2	3	5	1	9
Type 2/3	4	2	1	7
Type 3	1	1	11	13
Totals	20	9	13	42

χ^2 *P*-value < 0001.

scribed to FraX and non-mentally retarded control subjects. As mentioned above, only one normal control male, with FMRP type 1, showed a weak pattern. In contrast, 12 out of 13 males with FMRP type 3 (mostly with FM) displayed an abnormal 2D PAGE pattern, with the majority (11/12) displaying the intense pattern. Individuals with MOS and FMRP types 1, 2, and 2/3 showed a more diverse distribution. Of the approximately half of MOS by FMRP patterns (9/17) who displayed an abnormal 2D PAGE pattern, most of them (7/9) corresponded to the weak one. The overall chi-square analysis showed significant results ($P < 0001$).

FMRP Levels and 2D PAGE Patterns

When FMRP immunoreactivities (-ir) were measured and analyzed as continuous variables, we found a strong relationship between FMRP deficit and abnormal 2D PAGE pattern (Fig. 2). These analyses included 14 subjects with normal 2D pattern (one MOS, 13 controls), nine with the weak abnormality (one developmentally delayed, eight with FraX), and 12 with an intense pattern (all FraX). Consistent with our previous studies [Kaufmann et al., 1999], reductions in total FMRP-ir and HMW FMRP-ir, but not the 70 kd FMRP-like-ir, were highly associated with the abnormal 2D PAGE pattern. Both total FMRP-ir and HMW FMRP-ir were higher in individuals with normal 2D patterns and significantly lower (when compared to the former group) in males with intense abnormality ($P = 01$ and $P = 002$, respectively, by Bonferroni/Dunn corrections). Subjects with a weak pattern showed intermediate total FMRP-ir and HMW FMRP-ir levels which were not significantly different from either those with a normal or an intense pattern. Although 70 kd FMRP-ir displayed the same general trends observed for the other variables (Fig. 2), no significant differences were found when different patterns were compared.

Abnormal 2D PAGE Pattern Corresponds to Annexin-1

Examination of published [Shaw et al., 1999] and Web-posted databases (<http://www.expasy.ch/>, <http://www.expasy.proteome.org.au/>) [Hoogland et al., 1999] identified the abnormally expressed protein, in terms of MW (~37 kd) and pI, as annexin-1 (Anx-1), also termed (lipocortin-1) [Miele et al., 1988; Alldridge et al., 1999]. Anx-1 is a regulatory protein expressed in lymphocytes, macrophages [Reem and Yeh, 1984; Miele et al., 1988], and other cell types including keratinocytes, astrocytes, microglia and, perhaps, neurons [Fava et al., 1993; Eberhard et al., 1994; Naciff et al., 1996; Voermans et al., 1997; Savchenko et al., 2000]; it is involved in regulating phospholipid aggregation and mediating anti-inflammatory response [Alldridge et al., 1999] and other glucocorticoids effects [Jessop, 1999]. Although Anx-1's role in the CNS is still unclear, it may participate in local inflammatory responses [Savchenko et al., 2000] as well as in regulation of limbic

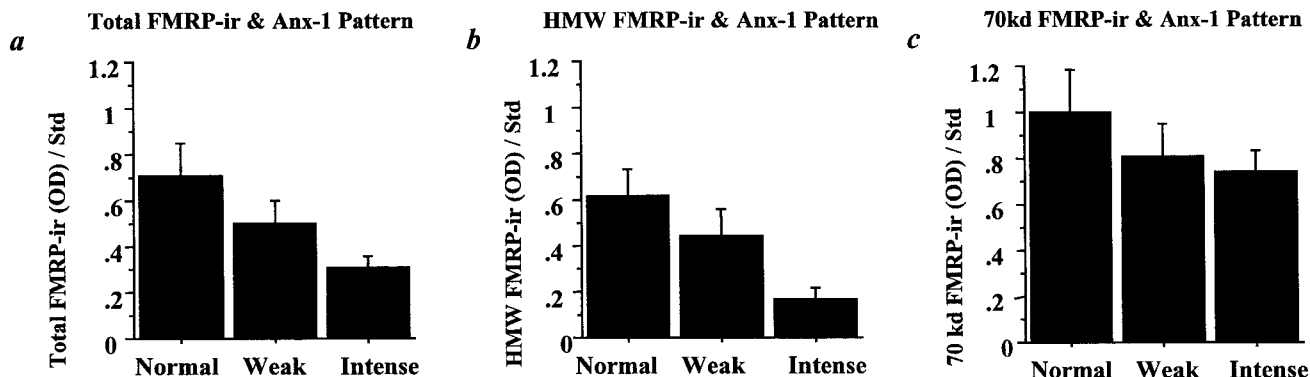


Fig. 2. Relationship between levels of FMRP-ir and abnormal 2D PAGE pattern. (a) Total levels of FMRP-ir were markedly different among the three 2D patterns, in direct proportion to the severity of the abnormality. There was a significant ($P=01$) reduction in total FMRP-ir in individuals with intense 2D pattern, when compared with those with a normal pattern. (b) Similar significant differences ($P=002$) were found in

comparisons of HMW FMRP-ir. (c) While levels of 70 kd FMRP-ir were higher in subjects with normal 2D PAGE patterns, the differences were not statistically significant. Bar graphs represent mean and one standard error. Immunoreactivity with respect to a standard was measured in optical density (OD) units.

system function [Voermans et al., 1997]. Confirmation of the identity of Anx-1 was carried out by immunoblotting, with specific monoclonal antibodies, of both 1D (not shown) and 2D PAGE (Fig. 3) gels as previously reported [Emmert-Buck et al., 2000]. As expected from the stained 2D gels, Anx-1 appears as a single, but rather broad, band in individuals with normal 2D PAGE pattern. Multiple spots, some of them fused, characterized Anx-1-ir in individuals with intense 2D PAGE abnormality (Fig. 3). An intermediate pattern of Anx-1-ir was found in subjects with a weak 2D PAGE abnormality (Fig. 3).

Qualitative vs. Quantitative Changes in Anx-1 in Fragile X Syndrome

Based on our observation of a more intense protein stain in gels displaying the intense 2D PAGE abnormality, and on our immunoblotting assays described above, we hypothesized that Anx-1 levels are increased as a function of FMRP deficiency. Therefore, we examined the relationships between Anx-1-ir levels and both FMRP deficiency and Anx-1 2D abnormality. A study of 18 subjects, evenly distributed across DNA control, MOS and FM categories, demonstrated that although Anx-1 levels were higher in males with FM

(Fig. 4a), there were not significant differences among the three groups (overall ANOVA $P=7$). Similar results were seen when individuals were grouped by FMRP type. Comparison of Anx-1-ir between the three 2D PAGE patterns, again, revealed higher levels in the group with intense 2D abnormality (predominantly FM), with the lowest level corresponding to those subjects with a weak abnormality (primarily MOS) (Fig. 4b). However, the differences were not statistically significant (overall ANOVA $P=2$).

Nature of the Anx-1 2D PAGE Abnormality

Anx-1 is a calcium-dependent phospholipid-binding protein, and a putative substrate of several major kinases, including protein kinase C [Fava et al., 1993]. Accordingly, Anx-1 contains a number of potential phosphorylation sites [Hall et al., 1993]. Although the aberrant 2D PAGE pattern suggests abnormal post-translational modification, other than phosphorylation (relatively no change in MW) [Black et al., 1986], we examined the effect of dephosphorylation on Anx-1 2D PAGE patterns. These assays were performed using changes in neurofilament phosphorylated epitope immunoreactivity as standards [Kaufmann et al., 2000]. When leukocyte homogenates were incubated

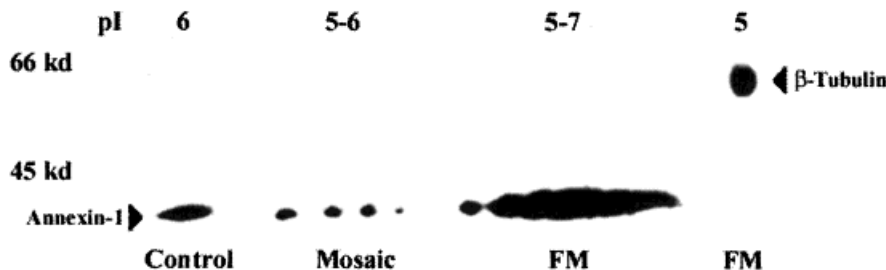


Fig. 3. Patterns of Anx-1 immunoreactivity after 2D PAGE. In correspondence with the 1-2 spots on silver-stained gels, control subjects (Cont) showed a single but broad immunoreactive spot. Subjects with mosaicism (mosaic) typically displayed 4-5 Anx-1-ir spots, as detected by 2D PAGE stained gels. Individuals with FM showed an intense and broad

Anx-1-ir band that represents the fusion of the 5-7 spots seen on stained gels. In order to demonstrate the specificity of the immunoblotting assays, immunoreactivity for another major leukocytic protein, β -tubulin is also depicted.

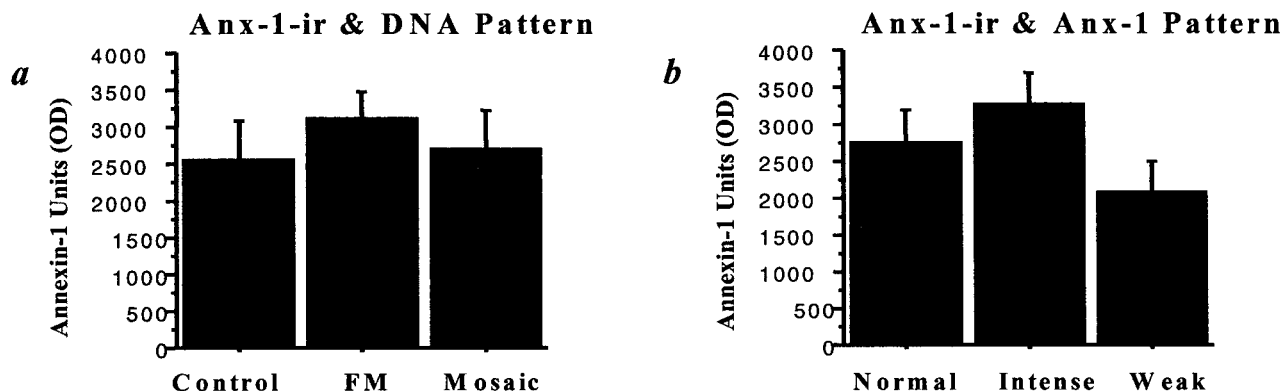


Fig. 4. Relationships between levels of Anx-1-ir and *FMR1* mutation and Anx-1 2D PAGE patterns. (a) Levels of Anx-1-ir, measured by immunoblotting, in individuals with normal, FM, and MOS DNA patterns. Note the relatively higher Anx-1-ir in the FM group, with lowest levels displayed in control subjects. The differences were not statistically significant. (b) Levels of Anx-1-ir in individuals with normal, weak, and

intense Anx-1 2D PAGE patterns. In agreement with Figure 4a, Anx-1-ir levels were moderately but not significantly higher in subjects with an intense 2D pattern. Bar graphs represent mean and 1 standard error. Immunoreactivity with respect to a standard was measured in optical density (OD) units.

with alkaline phosphatase, under conditions that markedly reduced phosphorylated neurofilament immunoreactivity (Fig. 5b), there were no obvious changes in Anx-1 2D patterns (Fig. 5a, b). Based on these results, and on the evaluation of previously published 2D patterns linked to posttranslational modifications [Black et al., 1986; Hall et al., 1993], we concluded that Anx-1 abnormality in FraX corresponds to abnormal acetylation.

DISCUSSION

The search for FMRP targets, which can explain the mechanisms by which the deficiency in the *FMR1* product leads to neurobehavioral dysfunction, has predominantly focused on identifying transcripts

bound to and regulated by FMRP [Bardoni et al., 1999; Ceman et al., 1999; Zhong et al., 1999; Sung et al., 2000]. Here we propose a novel approach: the use of 2D PAGE. This technique displays the entire proteome of a cellular or tissular sample [Black et al., 1986; Hoogland et al., 1999] and is, therefore, particularly suitable for studies in FraX, since FMRP is a protein involved in protein synthesis and not in RNA transcription [Jin and Warren, 2000]. Our study, to our knowledge, is the first to use 2D PAGE in FraX leukocytes, confirming the potential usefulness of this method. Through this procedure, we found that an abundant protein in leukocytes, Anx-1, is abnormally expressed in FraX as a function of FMRP deficit.

The specificity of this Anx-1 abnormality (to FraX) is supported by its strong association with both *FMR1*

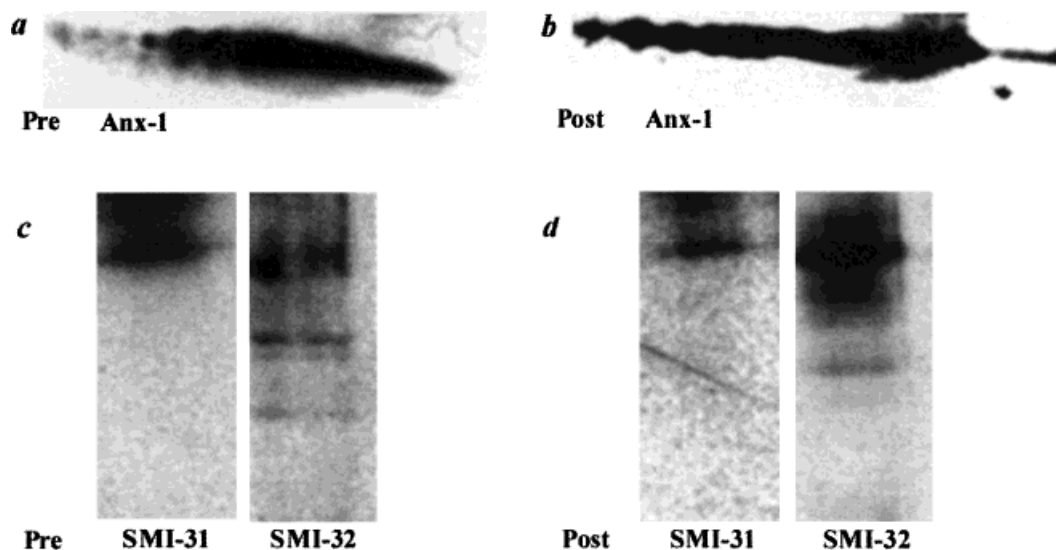


Fig. 5. Effect of dephosphorylation upon Anx-1 2D PAGE patterns. Leukocyte homogenates from an individual with FM (a) were subjected to dephosphorylation by incubating with alkaline phosphatase, prior to 2D PAGE. Note the lack of change in the leukocyte Anx-1-ir pattern after the enzymatic reaction (b). This contrasts with the reduction in phosphorylated

neurofilament (SMI-31-ir) and increase in non-phosphorylated neurofilament immunoreactivities (SMI-32-ir), detected in brain homogenates subjected simultaneously to the same protocol. c and d: Homogenates before and after dephosphorylation. Pre and Post refer to before and after incubation with phosphatase.

and FMRP patterns. Only one control showed the mild form of this Anx-1 abnormality. In contrast, individuals with FM and most severe FMRP reduction (FMRP type 3) almost invariably displayed the phenomenon. Moreover, levels of FMRP, particularly of the most prominent and probably most functionally relevant [Kaufmann et al., 1998] HMW isoform, were highly associated with the Anx-1 2D pattern. This relationship was emphasized by the presence of a gradation in the Anx-1 2D phenomenon. While males with FM showed predominantly the most intense Anx-1 abnormal 2D pattern, individuals with intermediate levels of FMRP-ir (MOS) also displayed the intermediate or weak 2D PAGE pattern. The abnormal Anx-1 2D patterns appear to be a part of a major disruption in Anx-1 expression, since Anx-1-ir levels were also slightly increased in those subjects with the intense 2D PAGE pattern.

In contrast to the clear relationship between FMRP deficit and Anx-1 abnormality is the uncertain nature of the 2D PAGE pattern. The 2D PAGE abnormality could be better characterized as a dysregulation than as a downregulation, as recently reported in esophageal cancer [Emmert-Buck et al., 2000]. Based on previous 2D PAGE studies [Black et al., 1986; Hall et al., 1993; Shaw et al., 1999], Anx-1 2D pattern most likely reflects an abnormality in this protein's posttranslational processing. Phosphorylation is the best characterized, both biochemically and functionally, posttranslational change experienced by Anx-1 [Fava et al., 1993; Alldridge et al., 1999]. The 2D pattern we describe in this study, without obvious increase in apparent MW, as well as our own dephosphorylation assays, do not support a modification in the phosphorylation status of Anx-1 in FraX. Taking into consideration earlier reports of posttranslational modifications in cytoskeletal proteins [Kaufmann et al., 2000], in particular changes affecting α -tubulin prior to its polymerization into microtubular structures [Black et al., 1986], Anx-1's multiple 2D spots may represent acetylation of additional residues. Although acetylation is a relatively less well-known posttranslational change of Anx-1, and our inference is purely based on the similarity between our data and previously published 2D patterns of other proteins, this posttranslational modification may be important for Anx-1's function in the CNS. Acetylation of the N-terminal 13 residues of Anx-1 [Hall et al., 1993] has been linked to its interaction with S100C [Lewit-Bentley et al., 2000; Rety et al., 2000], a novel member of the S100 calcium-binding protein family that is involved in inhibition of cell growth [Sakaguchi et al., 2000] and ATPase activity [Zhao et al., 2000]. The importance of N-terminus acetylation is emphasized by reports of similar protein-protein interactions affecting other members of the annexin family, such as the interaction between Anx-2 and p11, which has been implicated in membrane fusion processes [Lewit-Bentley et al., 2000; Rety et al., 2000]. Our finding of other groups of spots in FraX subjects, which resemble the abnormal Anx-1 2D pattern, also suggests that abnormal acetylation may be a more general disturbance in FraX. The exact nature of the Anx-1 2D PAGE

abnormality, as well as its relationship to aberrant acetylation, is currently under investigation in our laboratory.

The relationship between a defective function of Anx-1 and the FraX phenotype remains unclear. It is known, however, that in addition to its putative role in the regulation of peripheral [Reem and Yeh, 1984; Miele et al., 1988; Alldridge et al., 1999] and central [Knott et al., 2000; Savchenko et al., 2000; Melzer et al., 2001] inflammatory responses, Anx-1 also plays a critical role in pituitary function. Specifically, among other hormonal regulatory responses, Anx-1 mediates the inhibition exerted by glucocorticoids on the hypothalamo-pituitary-adrenal axis [Jessop, 1999]. Thus, the function of Anx-1 in the CNS appears to be linked to FraX since recent data show that cortisol levels, particularly after stress, are higher in males with FraX [Wisbeck et al., 2000]. This hormonal disruption has been postulated as having a role in socio-emotional aspects of the FraX neurobehavioral phenotype. Moreover, Anx-1 expression is selectively induced by exogenous glucocorticoids in brain regions (e.g., hippocampus) [Philip et al., 1997; Voermans et al., 1997] found to be involved in FraX [Kaufmann and Reiss, 1999]. The recently described neurotrophic role of astrocytic-origin Anx-1, which includes the promotion of neurite outgrowth and synaptogenesis of embryonic cortical neurons [Mizuno et al., 1998], may have implications for the dendritic anomalies that characterize FraX [Kaufmann and Moser, 2000]. Nonetheless, a direct assessment of Anx-1 expression in neural tissues from FraX patients is necessary in order to fully understand the significance of these findings for the FraX neurobehavioral phenotype.

In conclusion, we have found an abnormal pattern of Anx-1 expression in leukocytes from FraX subjects. This molecular abnormality, which seems to be directly related to FMRP deficiency, consists of an aberrant posttranslational modification (possibly, acetylation) that may lead to abnormal function of Anx-1 (i.e., protein-protein interaction). Defective Anx-1 could be responsible for some aspects of the FraX neurobehavioral phenotype, specifically those linked to the limbic system. These data also emphasize the feasibility of using 2D PAGE as a method for disclosing molecular abnormalities in FraX and other genetic disorders.

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